Community Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Molluscs, Cefas, Weymouth

Technical Report for Calendar Year 2009

Cefas Weymouth Laboratory,
Barrack Road, The Nothe,
Weymouth, Dorset, DT4 8UB, UK
Telephone: +44 (0) 1305 206600
Fax: +44 (0) 1305 206601
E-mail: fsq@cefas.co.uk
Website: www.crlcefas.org

Contents

Legal functions and duties 2
Introduction 2
1. Scientific advice and support 2
2. Co-ordination of activities of NRL network and provision of technical assistance and training 7
3. Proficiency testing and quality assurance 10
4. Confirmatory testing 14
5. Development of analytical methods 14

List of Annexes

Annex I - Work programme for the CRL for bacteriological and viral contamination of bivalve molluscs 2009. 16
Annex II - Resolutions of the 8th workshop of NRLs for bacteriological and viral contamination of Bivalve Molluscs, 2009. 20
Annex III - Agenda of the 8th workshop of NRLs for bacteriological and viral contamination of Bivalve Molluscs, 2009 24
Annex IV - Sanitary surveys- training workshop 2009 27
Annex V - Summary of participation amongst NRLs and others in CRL organised proficiency testing 29
Annex VI - Report on the whole animal bivalve shellfish ring trial: Enumeration of *Escherichia coli* and the detection of S*almonella* spp.. 30
Annex VII - Report on the Norovirus/Hepatitis A Ring Trial. 53
Annex VIII - RT 27 Norovirus and hepatitis A ring trial 41
Annex IX - RT 29 *Vibrio parahaemolyticus* 55

Community Reference Laboratory for Monitoring Bacteriological and Viral Contamination of bivalve Molluscs, CEFAS, Weymouth

Technical Report for Calendar Year 2009

Legal functions and duties

The functions and duties of the CRL are specified in Article 32 of Council Regulation (EC) 882/2004 (Official Journal of the European Communities No L165).

Introduction

The annual work programme for the CRL for 2009 was approved by the European Commission in December 2008. This report details consequential activities of the CRL according to the work programme 2009 (Annex I), additional tasks described under the resolutions of the 8th workshop of microbiological NRLs held in Palma de Mallorca, Spain (Annex II) and other responsibilities outlined in Commission Regulation (EC) 882/2004 for the calendar year 2009.

1. Scientific advice and support

To the European Commission.

The CRL provided advice to DG SANCO in the form of position papers, reports and briefing notes in the following areas:

- Classification of marine gastropods, echinoderms and tunicates across EU Member States.
- Scientific basis of 10% tolerances in class A and B live bivalve molluscs (LBM) harvesting areas.
- Equivalence of the 3 class plan for *Escherichia coli* in LBM (Codex STAN292/2008) and the 2 class plan in Commission Regulation (EC) No. 2073/2005.
- Scientific basis for the use of five-tube, three-dilution most probable number (MPN) in the enumeration of *E. coli* in LBM.
- Use and interpretation of Most Probable Number (MPN) tables.
- Considerations on the implications of the repeal of the Shellfish Water Directive (2006/113/EC) and implementation of the Water Framework Directive (2000/60/EC).
- CRL scientific opinion on the requirements of Commission Regulation (EC) No. 854/2004 with respect to sanitary surveys.
- Protocol for managing lack of collaboration by NRLs with Official Laboratories.
- Microbiological test requirements for frozen scallops.
- CRL opinion on the validation of an impedance method using BacTrac 4300 for enumeration of *E. coli* in LBM against the EU reference method for *E. coli* ISO TS 16649-3.

- Joint CRL report on Microbiology of food and animal feeding stuffs ISO SC9 and CEN TC275 WG6 Valencia 2009.
- Preliminary assessment to Unit 4 on the bilateral equivalency of LBM produced under EU and United States (US) systems.

The CRL continued to work with DG SANCO with respect to the publication of the Good Practice Guide to Microbiological Monitoring of Bivalve Harvesting Areas: Technical Application as a Commission Guidance document. The CRL also provided specialist assistance to the Food and Veterinary Office through the provision of national experts on missions to the US, Canada, Vietnam, China and Peru. The CRL provided TAIEX division with advice to assist in accreditation of microbiological testing of LBM in Albania.

Note. A full register of advice provided by the CRL to the European Commission and its divisions is available from the CRL co-ordinator on request.

To the EU NRL network, EFTA , accession and third country laboratories

In 2009 the CRL provided specific scientific advice to laboratories within the network with respect to interpretation of Commission Regulation (EC) No. 854/2004 on sanitary surveys; technical application of sanitary surveys; advice on the use and interpretation of MPN tables for use in Official Control testing for *E. coli* in LBM; accreditation of microbiological determinands including noroviruses; technical requirements for the use of PCR testing of foodstuffs, initial preparation of LBM samples; roles and responsibilities of NRLs; serotyping of Spanish *Vibrio parahaemolyticus*, and storage and use of reference culture collections (CRL *Campylobacter*). The CRL collaborated with NRL France in the validation to ISO 16140 of the impedance technique (Bactrac 4300) for enumeration of *E. coli* in LBM against the EU stipulated reference method (ISO TS 16649-3). And, provided substantial advice and guidance to The Netherlands in the ongoing validation of ISO 16649-2 (pour plate method) against ISO TS 16649-3. In 2009 additional supporting information and guidance for NRLs on comparative testing was generated including laboratory troubleshooting advice, guidance on performance assessment and follow-up activities.

The CRL has also worked closely with NRL Spain (Centro nacional de alimentacion, Madrid) to assist in delivery of the roles and responsibilities of NRLs as outlined in Article 33 of Commission Regulation (EC) No. 882/2004. The CRL provided significant support to NRL Denmark regarding attendance and presentation at a 2 day conference “Shellfish Sanitation” in Jutland in November 2009.

The CRL maintains very strong research collaborations with expert laboratories in the LBM sector in third countries. Notably in 2009 the CRL provided the outputs from norovirus and hepatitis A methods working group (see section 5 -Development of analytical methods), to testing laboratories in Australia (Victoria and South Australia), Japan, New Zealand, UK, US and Uruguay. This is indicative of the worldwide uptake of EU reference methods developed under the CRL remit.

Note. A full register of advice provided by the CRL is available from the CRL co-ordinator on request. EU NRL network, EFTA, accession and third country laboratories.

Other Scientific activities

CRL staff chaired or participated several ISO SC9 and CEN WG6 CEN "Microbiology of food and animal feeding stuffs" initiatives including:

- CEN/TC 275/WG6/TAG4 expert working group on viruses in foods developing a horizontal method for the detection of norovirus and hepatitis A in bivalve molluscs – CRL lead (see section 5).
- CEN/TC 275/WG6/TAG3 molecular methods expert working group on human pathogenic vibrios – CRL lead (see section 5).
- ISO TC34/SC9/WG3 "Microbiological validation" reviewing ISO 16140 for the validation of alternative methods.
- ISO TC34/SC9/WG4 "Proficiency testing".

The CRL presented the results of a wide range of research at the 7th International Conference on Molluscan Shellfish Safety (ICMSS) and 14th International Health Related Water Microbiology Conference covering correlation between quantitative real-time PCR results for norovirus and consumer illness; development of microbial source tracking in shellfish harvesting waters; evaluation of proficiency testing material for the detection of norovirus and hepatitis A virus; examination of food matrix effects on norovirus assays and murine norovirus-1 as a surrogate for human norovirus in the environment and bivalve shellfish. The CRL was represented on the ICMSS working group that produced recommendations for WHO, FAO and Codex on standardisation of *Vibrio* spp. methods. The CRL is also represented on the International Steering Committee of the ICMSS conference.

CRL Publications

In 2009 the CRL produced the following peer review publications and book chapters.

Baker-Austin, C., J V. McArthur., A. Lindell., M. Wright., J. Gooch,. L. Warner. J. Oliver and R. Stepanauskas (2009). Widespread antibiotic resistance in the marine pathogen *Vibrio vulnificus*. *Microbial Ecology*. 57:151-159..

Baker-Austin, C., J. Morris, J. A. Lowther, R. Rangdale, and D. N. Lees (2009). Rapid identification and differentiation of agricultural fecal contamination sources using multiplex PCR. *Letters in Applied Microbiology*, 49:529-532.

Baker-Austin, C., R. Rangdale, and D. N. Lees (2009). Developing microbial cource tracking methods for shellfish harvesting areas. *Shellfish News*.

Baker-Austin C., McArthur J. V., Lindell A. H., Wright M. S., Tuckfield R. C., Gooch J., Warner L., Oliver J. and Stephanauskas R. (2009) Multi-site analysis reveals widespread antibiotic resistance in the marine pathogen *Vibrio vulnificus*. Microb Ecol 57 (1) 151-159.

Lee R.J., Murray L., Catherine M., Amouroux I. (2009) The implementation and application of sanitary surveys in Europe Sixth International Conference on Molluscan Shellfish Safety. 247-255 New Zealand.

Lees D.N., Lowther J. and Rangdale R. (2009)International standardisation and quality assurance of methods for detection of human pathogenic viruses - a review of the issues and EU progress towards adoption of virus standards. Sixth International Conference on Molluscan Shellfish Safety. 3-16, New Zealand.

Murray L. H. And Lee R. J. (2009) Risk Management in Shellfisheries in Shellfish safety and quality edited by Sandar E. Shumway and Gary E. Rodrick, Woodhead Publishing in Food Science, Technology and Nutrition.

Rangdale R. E. (2009) Sewage borne pathogens associated with bivalve shellfish . Microbiologist, Society for Applied Microbiology 10(1); 29-32.

Rangdale R. E. (2009) Prevalence and potential pathogenicity of *Vibrio parahaemolyticus* in chinese mitten crabs (*Eriocheir sinensis*) harvested from the river thames estuary, England . Journal of food protection; 72; 1;60-66.

Rangdale R. E. (2009) Viruses and bivalve shellfish. International Journal of Food Microbiology 59 81-116.

Wagley S., Koofhethile K., Badger Wing J., and Rangdale R. (2009) *Vibrio parahaemolyticus* in the United Kingdom and the first identification of the pandemic 03:K6 serotype. Sixth International Conference on Molluscan Shellfish Safety, 177-184, New Zealand.

Roque A., Lopez-Joven C., Lacuesta B., Elandaloussi L., Wagley S., Furones M.D., Ruiz-Zarzuela I., I de Blas I., Rangdale R. E. , and Gomez-Gil B. (2009) Detection and Identification of *tdh*- and *trh*-Positive *Vibrio parahaemolyticus* Strains from Four Species of Cultured Bivalve Molluscs on the Spanish Mediterranean Coast . Applied and environmental microbiology 1 December 2009; Vol. 75, No. 23 7574-7577.

Copies of CRL publications can be obtained from the CRL co-ordinator on request.

2. Co-ordination of activities of NRL network and provision of technical assistance and training (including third countries)

Designation of NRLs and co-operation with the CRL

The CRL encourages full cooperation and participation by NRLs in the activities of the network as outlined in Article 32 of Commission Regulation (EC) No. 882/2004. To assist in co-ordination activities a list of designated NRLs is updated annually. This information is published on the CRL website www.crlcefas.org

Designated NRLs in Member States, EFTA and Accession states in 2009.

Member State	Laboratory
Austria	Austrian Agency for Health and Food Safety, Institute for Food Control, AGES-LMU Wien, Abt. Mikrobiologie, Spargelfeldstraße 191 A-1220 Wien.
Belgium and Luxembourg	Scientific Institute of Public Health (IPH), Rue J. Wytsmanstraat, 14, 1050 Brussels.
Bulgaria	National Diagnostic and Research Veterinary Institute, Pencho, Slaveikov, 15 BG - 1606 Sofia
Croatia[1]	No NRL designated
Cyprus	No NRL designated
The Czech Republic	State Veterinary Institute, Rantirovska 93,Jihlava, CZ - 58605 Jihlava
Denmark	Department of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark, Morkhoj Bygade 19 DK 2860 Soborg.
Estonia	No NRL designated
FYR[1]	No NRL designated
Finland	Finnish Food Safety Authority Evira, Research Department Microbiology Unit, Mustialankatu 3, FI-00790, Helsinki
France	IFREMER, Departement Microbiologie et phycotoxines, Centre de Nantes, Rue de l'Ile de'Yeu, BP 21105, 44311 Nantes Cedex 3.
Germany	Federal Institute for Risk Assessment, Diedersdorfer Weg, D-12277, Berlin.
Greece	Institute of Food Hygiene of Athens, Neapoleos 25, 15310 Ag. Paraskevi, Attiki, Athens.
Hungary	Central Veterinary Institiute, 1149 Budapest, Tábornok u.2.
Iceland[2]	Matis, Matvaelarannsoknir Islands, Skulagotu 4, 101 Reykjavik.
Ireland	Marine Institute, Orinville, Oranmore, Galway
Italy[3]	Centro di Referenza Nazionale per il controllo microbiologico e chimico dei molluschi bivalvi vivi, Via Cupa di Posatora3, 60100, Ancona. Dipartmento di Sanita Pubblica Veterinaria e Sicurezza Alimentare, Viale Regina Elena 299, Roma.
Latvia	State Veterinary Medicine Diagnostic Centre (SVMDC), Food and Veterinary Service Lejupes str.3, Riga.
Lithuania	National Food and Veterinary Risk Assessment Institut, J.Kairiukscio 10, LT-08409 Vilnius.
Malta	No NRL designated

Member State	Laboratory
The Netherlands	National Institute for Public Health and the Environment (RIVM), PO Box 1A van, Leeuwenhoeklaan 9, 3720 BA, Bilthoven.
Norway[2]	The Norwegian School of Veterinary Science, Institute for Pharmacology, Microbiology and Food Hygiene, P.O Box 8146 Dep, 0033 Oslo.
Poland	National Veterinary Research Institute, Panstwowy Instytut, Weterynaryjny, Al.Partyzantów 57, PL - 24-100 Pulawy.
Portugal	Instituto de Investigacao das Pescas e do Mar (IPIMAR), Avenida Brasilia, 1449-006 Lisboa.
Romania	Institute for Diagnosis and Animal Health, Str. Dr. Staicovici, nr. 63 Sector 5, cod 050557, Bucuresti.
The Slovak Republic	State Veterinary and Food Institute, Janoskova 1611/58 Ministry of Agriculture, SK - 02601 Dolny Kubin.
Slovenia	National Veterinary Laboratory, Gerbiceva 60, SI – 1000, Ljubljana.
Spain	Centro Nacional de Alimentacion, Agencia Espanola de Seguridad Alimentaria, E-28220 Majadahonda,Madrid.
Sweden	National Food Administration, P.O. Box 622, 75126 Upsalla.
Turkey	No NRL established
United Kingdom	Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB.

[1] Accession State
[2] Member of the EFTA
[3] 2 NRLs established (microbiological monitoring and virology)

Website

The CRL website www.crlcefas.org is one of the primary means of dissemination of information and is used extensively by stakeholders. Following the annual website review in 2009 the content was revised and text simplified to improve accessibility. The public domain sections of the website comprise details of the designation and contact details of the NRL network, the CRL comparative testing programme and documents supporting laboratory quality assurance. The **Information Centre** (which requires registration) now contains the following sections:

- CRL work programmes and technical reports.
- Information relating to workshops of NRLs.
- CRL publications.
- Laboratory standard operating procedures (SOPs).
- Comparative testing reports and guidance documents.
- Sanitary surveys.

User statistics for the CRL website for 2009 are given in Figure 1. In 2009 users registered over 1800 visits per month representing a 15% rise in visitor usage compared with 2008.

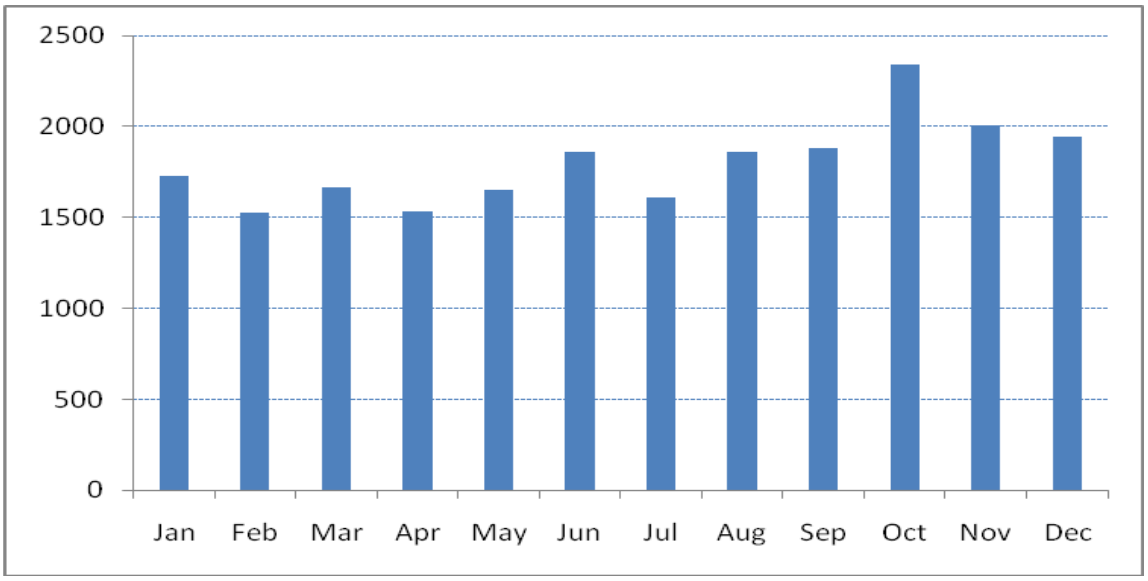


Figure 1. Visitor activity by months in 2009.

Note. Figure 1 shows the number of individual visitor sessions recorded on the website per month between January and December 2009. A session reports individual users for any given time interval. Sessions are tracked per IP address and must register at least one hit to be included. A hit is defined as any request for data such as a web page, bitmap, CGI gateway or file.

NRLs workshop May 12-14th Palma de Mallorca, Spain

The workshop of European NRLs was held at the Hotel Tryp Bosque, Palma de Mallorca, Spain. Forty-one experts representing NRLs from 19 Member States, NRLs from EFTA countries Iceland and Norway also attended. In 2009 a dedicated session focusing on *Vibrio* spp. associated with LBM was convened. As agreed at the previous workshop in 2008, internationally recognised experts from the United States, Chile, Japan and Spain were invited to present current research methods and mitigation strategies. A full report of the 8th workshop including copies of all of the presentations and associated meeting papers are available on the CRL website (www.crlcefas.org) or directly from the CRL co-ordinator. Thirty-seven resolutions were passed by NRL delegates. The workshop resolutions are included as Annex II of this report and the workshop agenda as Annex III.

Training and technical assistance

Formal training workshops

The CRL hosted a training workshop on the application of sanitary surveys as required under Commission Regulation (EC) No. 854/2004 in October 2009. Delegates from seven Member States (representing 9 autonomous or semi-autonomous regions) attended the four day event. As

in previous training courses in this field the intent was to deliver training to personnel responsible for implementation of the Regulation. Attendees were provided with theoretical and group based practical training that included a full shoreline survey. The training programme is included as Annex IV of this report. The course programme is now being developed as a web-based training module on the CRL website.

Additional ad hoc training and study visits

The CRL organised study visits in its area of competence for technical staff from the US FDA, Gulf Coast Seafood Laboratory, Alabama, United States – comparative methods assessment norovirus; Public Health Laboratory Services, University of Melbourne, Victoria, Australia – quantitative determination of hepatitis A in BMS; PIRES-SARDI, South Australia – detection and enumeration methods for pathogenic *Vibrio* spp.; ESR, Kenepuru, New Zealand - quantitative determination of noroviruses in BMS and King's College, London, UK - quantitative determination of *V. vulnificus* and *V. parahaemolyticus* in BMS.

Provision of technical assistance and reference materials

Additional technical assistance was supplied to NRLs and others in the form of provision of CRL standard operating procedures (SOP), laboratory protocols, MPN tables, ISO 17025 accreditation requirements, virus risk assessments, storage and usage of reference strains. In addition, the CRL provided technical advice and assistance to the DG SANCO initiative "Better Training for Safer Food". Reference control materials in the form of norovirus (GI, GII) and hepatitis A (HAV) plasmids, bacterial cultures, HAV tissue culture supernatant, and NoV GI and GII faecal suspensions as lenticules were distributed to NRLs in Denmark, Slovakia, Italy (Ancona), Norway and laboratories in South Korea and New Zealand.

Note. A full register of requests and CRL responses can be obtained from the CRL co-ordinator on request.

3. Proficiency testing and quality assurance

Participation of MS NRLs, accession and third countries in CRL organised proficiency testing (PT) in 2009 is summarised below and tabulated in Annex V. PT (ring trial) reports are presented in Annexes VI, VII and VIII. PT reports are presented at the annual workshop of NRLs and are also available on the CRL website www.crlcefas.org

E.coli / *Salmonella* External Quality Assurance Scheme (EQA)

The CRL collaborates with the UK Health Protection Agency (HPA) on the application of a Shellfish External Quality Assurance (EQA) Scheme for proficiency testing among laboratories analysing BMS. The Shellfish EQA scheme is targeted at analysis of the statutory determinants *E. coli* and *Salmonella* spp. in BMS and is the primary means of measuring performance by NRLs. Participation by NRLs is given in Table 1. Rolling performance assessments for 3 EQA distributions to March 2010 are included in this report and summarized in Tables 2 and 3. A full report of laboratory performance is available from the CRL co-coordinator.

Table 1. Participation in the CRL/HPA EQA Shellfish scheme by NRLs 2009

	Participation in Shellfish EQA for	
Member State	***E. coli***	***Salmonella***
Austria	Yes	Yes
Denmark	Yes	Yes
Finland	Yes	Yes
France	Yes	Yes
Germany	Yes	Yes
Greece[a]	Partial	Partial
Ireland	Yes	Yes
Italy 1	Yes	Yes
Italy 2	Yes	Yes
Lithuania	Yes	Yes
Netherlands	Yes	Yes
Poland	Yes	Yes
Portugal	Yes	Yes
Romania	Yes	Yes
Slovenia	Yes	Yes
Spain	Yes	Yes
Sweden	Yes	Yes
United Kingdom	Yes	Yes
ETFA		
Norway	Yes	Yes

[a] laboratory only participated in one distribution

NRLs for Belgium, Bulgaria, The Czech Republic, Latvia and The Slovak Republic did not take part in the scheme. Lack of participation in CRL organized activities has been addressed through phase 1 of the Commission protocol for lack of collaboration and/or underperformance with CRLs. In addition NRL participation in comparative testing will be addressed at the workshop of NRLs in Ancona 2010.

[Facing page - Tables 2 and 3 Rolling performance assessment for NRLs for *E. coli* and *Salmonella* spp. Distributions in 2009/10].

Table 2	Distribution								
	SF032		SF033		SF034		Max pos.score	Cumulative score	%
Lab no.	SF0072	SF0073	SF0074	SF0075	SF0076	SF0077			
121	12	12	12	12	12	12	72	72	100
391	12	12	12	12	12	12	72	72	100
403	12	12	12	12	12	12	72	72	100
493	NE	NE	NE	NE	12	12	24	24	100[a]
583	12	9	12	12	2	2	72	49	68[b]
593	12	12	12	12	2	2	72	52	72
596	12	4	12	12	12	12	72	64	89
597	12	12	12	10	12	12	72	70	97
601	12	12	12	12	12	7	72	67	93
604	12	12	12	12	12	12	72	72	100
649	12	12	12	12	12	9	72	69	96
651	NR	NR	12	12	12	12	72	48	67[ab]
653	12	12	12	12	12	9	72	69	96
658	12	12	12	12	12	12	72	72	100
701	12	12	7	7	12	12	72	62	86
703	12	12	12	12	12	12	72	72	100
715	12	12	12	12	12	12	72	72	100
718	12	12	12	12	12	12	72	72	100
744	12	12	9	12	12	12	72	69	96

[a] Laboratories not subject to full performance assessment; [b] Laboratories contacted as performance assessment <70%..

Table 3	Distribution								
	SF032		SF033		SF034		Max pos. score	Cumulative score	%
Lab no.	SF0072	SF0073	SF0074	SF0075	SF0076	SF0077			
121	2	2	2	2	2	2	12	12	100
391	NE	NE	2	2	2	2	8	8	100[a]
403	2	2	2	2	2	2	12	12	100
493	NE	NE	NE	NE	2	2	4	4	100[a]
583	2	2	2	2	2	2	12	12	100
593	2	2	2	2	2	2	12	12	100
596	2	2	2	2	2	2	12	12	100
597	2	2	2	2	2	2	12	12	100
601	NE	NE	NE	NE	2	2	4	4	100[a]
604	2	2	2	2	2	2	12	12	100
649	2	2	2	2	2	2	12	12	100
651	NR	NR	2	2	2	2	12	12	100[a]
653	2	2	2	2	2	2	12	12	100
658	2	2	2	2	NE	NE	8	8	100[a]
701	2	2	2	2	2	2	12	12	100
703	2	2	2	2	2	2	12	12	100
715	2	2	2	2	2	2	12	12	100
718	2	2	2	2	2	2	12	12	100
744	2	2	2	2	2	2	12	12	100

E. coli and *Salmonella* spp. whole bivalve mollusc ring trial – RT31

In October 2009 the CRL organised a PT distribution using bivalve mollusc matrix samples to examine initial sample preparation, preparation of dilutions and matrix effect i.e. aspects not challenged by using the laboratory constructed samples within the CRL/HPA EQA scheme. Thirty-three laboratories participated (20 MS NRL and 15 Official Control laboratories). A full report of this PT is included as Annex VII.

Participants examined samples of Pacific oysters (*Crassostrea gigas*) for the statutory determinands (*E. coli* and *Salmonella* spp). Reported *E. coli* MPN values were compared to the median of all participants' results. Upper and lower acceptability limits were calculated as the participants' median ±3 theoretical standard deviations (SD) and ±5 SD (≈99% and 99.9% confidence intervals respectively). Performance assessment was according to the CRL/HPA EQA scheme for a single distribution, with modifications to reflect replicate analyses of a single sample.

All participating laboratories returned results for *E. coli* and *Salmonella* spp. Ninety-one percent (91%) of laboratories returned duplicate *E. coli* MPN results within the expected range for *E. coli*. Where information was provided laboratories cited ISO TS 16649-3 (Anon 2005) or a derivative of this as their laboratory method for enumeration of *E. coli*. This is indicative of the wide uptake of the reference method amongst NRLs and testing laboratories.

All laboratories returned the expected result for *Salmonella* spp.. Eighteen laboratories used the EU specified reference method for detection of *Salmonella* spp. (ISO 6579). However, 3 laboratories reported use of alternative methods that were not validated according to ISO 16140. The use of reference methods or suitably validated alternatives will be addressed through a dedicated session at the next workshop of NRLs.

Norovirus and Hepatitis A ring trial

The CRL distributed stabilised NoV (GI and GII derived from faecal material) and HAV (tissue culture HM175 strain) to 27 laboratories (17 MS NRLs, 4 in-country testing laboratories, 2 EFTA NRLs, and 4 third country laboratories including laboratories in New Zealand, Canada, South Korea and China). Samples contained moderate to low titre of virus to mimic environmental contamination levels. A full report of performance is included as Annex VIII (RT27). In brief, approximately a third of participating laboratories obtained intended results for all samples, as determined by CRL reference designations. For individual viruses, 41%, 48% and 72% of laboratories returned intended results for norovirus GI, GII and hepatitis A respectively. The false positive reporting rates for GI, GII and HAV were 4%, 6% and 6% respectively. The false negative

reporting rates for GI, GII and HAV were 11%, 6% and 11% respectively. Eighteen laboratories returned semi-quantitative data expressed as C_t values with 7 laboratories returning quantitative data expressed as detectable genome copies per lenticule. This year 96% of laboratories returned either fully or semi-quantitiatve results compared with 58% in 2008. This represents a substantial increase in the use of quantitative procedures for virus determination and a move away from presence/absence testing observed in previous comparative testing.

Vibrio parahaemolyticus ring trial

The CRL distributed four samples for detection of total and potentially pathogenic *V. parahaemolyticus* in March 2009. Twenty-two laboratories registered for the scheme, 12 NRLs and 10 testing laboratories in MS and third countries (including Canada, Chile, Croatia, Iceland and New Zealand). Forty-one percent of laboratories returned results corresponding to CRL expected results for presence / absence of *V. parahaemolyticus*. Eleven laboratories used methods that enabled detection of the pathogenicity markers of *V. parahameolyticus* (*tdh* and *trh*). The majority of laboratories applying these tests assigned the presence or absence of both *tdh* and *trh* in accordance with the CRL tested results. A number of standard methods (ISO, NMKL, BAM) and non-standard methods were in use for identification and enumeration of *V. parahaemolyticus* and for detection of pathogenic principles. The outcomes of this ring trial highlight the urgent need for standardization of fit-for-purpose methods for the detection and enumeration of potentially pathogenic *Vibrio* spp. for use in European bivalve shellfish. A full report of laboratory performance is included as Annex IX.

4. Confirmatory testing

During 2009 the CRL maintained accreditation to ISO 17025 for the following methods and associated procedures:

- Examination of shellfish for *Salmonella* spp.
- Enumeration of *Escherichia coli* in bivalve molluscan shellfish.
- Detection of *V. parahaemolyticus* in bivalve molluscan shellfish.
- FRNA bacteriophage enumeration bivalve molluscan shellfish.

5. Development of analytical methods

The CRL continued to undertake extensive practical research and development in support of initiatives at CEN and ISO Microbiology of food and animal feeding stuffs (ISO SC9 and CEN TC275 WG6).

Viruses in food (TAG4)

In 2009 the CRL continued to chair the CEN/TC 275/WG6/TAG4 expert working group on viruses in foods developing a horizontal method for the detection of norovirus and hepatitis A in bivalve molluscs. All outstanding technical issues relating to the proposed standard for quantitative determination of norovirus and hepatitis A were resolved and the method completed. The quantitative part of this draft standard was presented at CEN WG6 in the Spring of 2009. Comments from the preliminary technical consultation were addressed and the draft standard (TC34/SC9 N1065) Microbiology of food and animal feeding stuffs - Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantitative determination is on course for publication as an ISO standard by 2012. Part 2: Method for qualitative detection will be formally launched at the Spring meeting of SC9/WG6 in 2010. As designated project leader under CEN "Methods on analysis of foodstuffs concerning food hygiene" (M/381) in support of EU Food Hygiene Regulations, the CRL awaits progression on formal validation. Throughout 2009 the method was in routine use at the CRL and preparation for re-accreditation to ISO 17025 is underway.

Human pathogenic Vibrio spp. (TAG3)

The CRL continues to play an active role in research and development in the area of marine *Vibrio* spp. associated with bivalve shellfish. As project leader for *Vibrio* spp. the CRL has continued to work towards molecular based approaches to enable the enumeration and rapid detection of pathogenic strains of *V. parahaemolyticus* and *V. vulnificus* directly from seafoods.

Development of virus reference materials

A pilot study to assess the development of reference materials in matrix samples was initiated in collaboration with JRC IRMM, Geel, Belgium. Results will be reported in due course.

Interpretation of RT-PCR data

The aim of this work is to assist in interpretation of real-time PCR results for the detection of norovirus in LBM. The work examines stability of norovirus RNA in seawater and oysters, using *inter alia*, a cultivable norovirus surrogate (murine norovirus). Methods are under development for the detection of murine norovirus directly in LBM using molecular and tissue culture techniques. The results of this programme will assist in the understanding of the meaning of data generated from norovirus tests with respect to the risk to the consumer.

Annex I – Work programme for the CRL for bacteriological and viral contamination of bivalve molluscs 2009.

WORK PROGRAMME FOR THE CRL FOR BACTERIOLOGICAL AND VIRAL CONTAMINATION OF BIVALVE MOLLUSCS, 2009

LEGAL FUNCTIONS AND DUTIES

The functions and duties of the CRL are specified in Article 32 of Regulation (EC) No 882/2004 (Official Journal of the European Communities No L 165 of 30.4.2004).

In the 2009 work programme year 27 Member States and 3 candidate countries (Croatia, Turkey and Former Yugoslav Republic of Macedonia) are considered eligible for CRL assistance and invited to participate in CRL organised training programmes, ring trials/external quality assessments schemes etc. The full integration into the European Union of recent accession Member States continues to be a priority area, and is facilitated via the provision of additional advice, training and assistance.

WORK PROGRAMME, 2009

1. Scientific advice and support

1.1. Assist DG Sanco in functioning of Community food hygiene legislation, e.g. drafting guidance documents, consideration of analytical tolerances, etc.

1.2. Participate in relevant EU and International scientific committees (ISO/CEN, WHO/FAO, ICMSS etc). In 2009 the CRL will:

- Continue to chair and co-ordinate the activities of the CEN/TC 275/WG6/TAG4 developing a CEN standard for detection of norovirus and hepatitis A in foodstuffs, including bivalve molluscs (see resolution 23, 7th workshop of NRLs).
- Lead and co-ordinate the activities of CEN/TC 275/WG6/TAG3 in the elaboration of molecular based enumeration methods for pathogenic marine vibrios in bivalve shellfish (see resolution 28, 7th workshop of NRLs).
- Participate in ISO/TC34/SC9/WG3 working group on validation of methods (revision of EN ISO 16140) to include the elaboration of ISO technical report on recommendations for establishing/revising reference methods.
- Participate in working group CEN/TC 275/WG6/TAG6 on sampling methods to include recommendations on sampling bivalve molluscan shellfish.

1.3. Assist DG Sanco with specialist assistance in relation to food and veterinary inspections of Member States, Accession Countries and Third Countries and with other trade issues (e.g. equivalency negotiations) as they arise.

1.4. Support the industry initiative SUMO through technical oversight of the work programme.

1.5. Co-operate with, and assist DG TAIEX in the provision of training and advice to Accession Counties.

1.6. Undertake CRL missions in support of the above activities.

- During 2009 missions are foreseen in relation to the annual meetings of ISO and CEN (up to 2 missions); the CEN/TAG4 working group on viruses in food (2 missions); CEN/TAG3 working group on vibrios (2 missions); ISO/WG3 working group on validation of methods (2 missions) and up to 3 missions in support of NRLs and DG Sanco activities.

1.7. Participation in relevant international scientific conferences, e.g. International Conference on Molluscan Shellfish Safety, France, June 2009, and the IWA Health-Related Water Microbiology Conference, Greece, September 2009, Interstate Shellfish Sanitation Conference, US, August 2009.

1.8. Participate as a member of the steering committee in the ICMSS' international forum on harmonisation of approaches to bivalve shellfish sanitation, including standardisation of methodologies for indicator organisms, and human pathogenic viruses and bacteria.

2. Co-ordination of activities of NRL network and provision of technical assistance and training

2.1. Participate in annual CRL Directors co-ordination meeting and other CRL co-ordination meetings/workshops as appropriate

2.2 Organise, host, and participate in the eighth annual NRL workshop, produce resolutions and other workshop outputs (May 2009, CRL Weymouth). To include CRL administrative assistance.

2.3 Undertake CRL activities and commitments agreed in resolutions at annual workshops (as posted on www.crlcefas.org).

2.4 Organise specialist, targeted, practical training for NRLs, MS competent authorities and the FVO on sanitary surveys - in accordance with the requirements of 854/2004 on official controls.

2.5 Complete follow-up activities associated with the joint EU/US FDA workshop on implementation and approaches to sanitary surveys.

2.6 Supply specialist information and advice on bacteriological and viral methods to NRLs (particularly new MS NRLs and accession countries), Official Control testing laboratories, and third county laboratories. To include assistance on implementation of methods, accreditation to IEC ISO17025, validation of alternative methods according to ISO16140, provision of CRL SOPs and transfer of other technical information.

2.7 Provide specialist training and/or training courses to NRLs, accession country NRLs and others in relation to analyses of *E. coli*, *Salmonella* spp., *Vibrio* spp., FRNA bacteriophage, Norovirus, hepatitis A virus and other aspects of bivalve shellfish hygiene as required.

2.8 Continue to update and improve the CRL website (www.crlcefas.org) as a primary means of dissemination of information to NRLs and others.

3 Ring trials, comparative testing and quality assurance

3.1 Organise comparative (proficiency) testing for NRLs for *E.coli* and *Salmonella* spp. in bivalve molluscs via the CRL/HPA shellfish EQA scheme (see resolution 14, 7th workshop of NRLs). Analyse results, produce report, advice and recommendations (by May 09).

3.2 Organise Norovirus and hepatitis A ring trials (see resolution 21, 7th workshop of NRLs). Analyse results, produce report and recommendations (by May 09).

3.3 Undertake *Vibrio parahaemolyticus* ring trials appropriate for methods enabling enumeration of pathogenicity principles (thermostable direct and thermostable direct related haemolysins) (see resolution 27, 7th workshop of NRLs). Analyse results, produce report and recommendations (by May 09).

3.4 Provision of EQA material, methods of analysis for FRNA bacteriophage (see resolution 20, 7th workshop of NRLs)

3.5 To challenge aspects of the *E. coli* and *Salmonella* spp. methods not covered by the standard shellfish EQA scheme organise a whole animal ring trial (see resolution 17, 7th workshop of NRLs)) for NRLs, the scheme will be extended to selected Official Control Laboratories. Analyse results, produce report, advice and recommendations (by May 09).

3.6 Prepare stable reference material using biological carriers for norovirus and Hepatitis A (see resolution 22, 7th workshop of NRLs). Perform homogeneity and stability analyses. Distribute data and LENTICULES to NRLs for use as control material on request.

4 Confirmatory testing

4.1 Maintenance of CRL laboratory competence and expertise on analytical methods for monitoring virological contaminants of bivalve molluscs (Norovirus and hepatitis A virus).

4.2 Maintenance of CRL laboratory competence and expertise on analytical methods for monitoring bacteriological contaminants of bivalve molluscs (*E.coli*, *Salmonella* spp., FRNA bacteriophage, marine vibrios). To include maintenance of IEC ISO 17025 accreditation of enumeration of *E. coli* and FRNA bacteriophage and the detection of *Salmonella* spp. and *Vibrio parahaemolyticus*.

4.3 Contribution to costs of the maintenance of CRL capability to perform analysis for marine vibrios in bivalve molluscs other than *V. parahaemolyticus.*

4.4 Performance of above tests on outbreak material or on occasion of disputed test results (on request of DG Sanco).

5 Development of analytical methods (undertaken at CRL)

5.1 Contribution as the project leader towards the validation of the TAG4 reference method for the detection of viruses in food (CEN/TC 275/WG6/TAG4).

5.2 Contribution as the project leader towards the elaboration and validation of the TAG3 molecular based standard for the detection of potentially pathogenic vibrios in foodstuff, including bivalve shellfish using molecular methods - both nucleic acid hybridisation and real time PCR approaches.

5.3 The existing *E. coli* enumeration reference method ISO TS 16649-3 specified in Commission Regulation (EC) No 2073/2005 is published as a technical specification with an expiry of December 2008. It is proposed that the CRL request that ISO SC9 grant an extension for the TS for a further 2 years. In addition, in collaboration with the NRL network, work to generate data enabling adoption as a full horizontal standard should be undertaken. Thus preventing the withdrawal the *E. coli* reference method.

5.4 It is proposed that limited research to assist in the clarification of the significance of quantitative PCR results for norovirus in bivalve molluscan shellfish in terms of public health risk is undertaken.

Annex II – Resolutions of the 8th workshop of NRLs for bacteriological and viral contamination of bivalve molluscs, 2009.

Resolutions of the 8th workshop of Microbiological NRLs for Bivalve Molluscs, Palma de Mallorca, Spain, 12-14th May 2009.

Microbiological methods – microbiological monitoring

1. NRLs supported the 3 class plan approach for testing compliance with the *E. coli* MPN criteria in live bivalve molluscs destined for direct human consumption (n=5, c=1, m=230, M=700 E. coli MPN/100g, according to ISO TS 16649-3) contained within Codex (CODEX STAN 292-2008). NRLs considered this provided a more accurate determination of the sanitary quality of a batch and was standard practice in food microbiology eg determination of *Salmonella* spp. in bivalve molluscs placed on the market.

2. Further to the above, the CRL agreed to seek further advice and draft a paper for circulation to NRLs and DG SANCO on the statistical equivalence in terms of public health risk of the existing class 2 plan for live bivalve molluscs placed on the market as specified in Commission Regulation (EC) No. 2073/2004 and the 3 class plan under Codex (CODEX STAN 292-2008).

3. NRLs identified that the 3 class plan under Codex (CODEX STAN 292-2008) applied only to end products (i.e. live bivalve molluscs destined for direct human consumption). The Codex standard and approach (3 class plan) does not apply to monitoring of harvesting areas and is not appropriate for this application.

4. The workshop noted the publication of Commission Regulation (EC) No 1021/2008 establishing a 10% tolerance for class B areas and with a maximum result upper limit of 46,000 MPN *E. coli*/100g.

5. Further to the above NRLs noted that compliance with the existing requirement for class A areas (100% compliance with≤230 *E. coli* MPN/100g) was not statistically practicable and not performed in practice. NRLs noted that it was similarly necessary to introduce a tolerance for class A areas in order to carry out the classification requirements under EU Regulation 854/2004 in a scientifically robust way.

6. The CRL agreed to recirculate previous considerations of possible tolerance criteria for class A areas for consideration by the Commission and NRLs.

Microbiological methods – statutory determinands

7. With regard to ISO TS 16649-3 (*E. coli* reference method) the CRL confirmed that a letter had been sent to ISO SC9 requesting an extension for an additional 3 years. In addition, it was noted that it would be preferable to provide additional data for matrices other than bivalve molluscs to facilitate its adoption as a full ISO standard method. The CRL agreed to circulate a protocol for further collaborative testing to support this on request.

8. The CRL confirmed that an amendment to ISO 7218:2007 to include probability categories for 5x3 MPN tables had been proposed. The network would be informed of progress in due course.

Microbiological methods –validation and use of alternative methods

9. The workshop noted the progress, reported by NRL The Netherlands, on the expert laboratory study aspects validation of ISO 16649-2 against the stipulated EU reference method for

enumeration of *E. coli* in live bivalve molluscs (ISO TS 16649-3). The workshop commented that it would be important to establish comparative data for an environmentally relevant range of naturally contaminated samples.

10. Further to the above the workshop noted the request, transmitted by NRL The Netherlands, for NRL participation in the forthcoming collaborative study for this validation. Laboratories were encouraged to communicate their interest in taking part in the interlaboratory trial to NRL The Netherlands.

11. The workshop noted the completion by NRL France of the validation according to ISO 16140 of the impedance method (Bactrac 4300) against the stipulated EU reference method for enumeration of *E. coli* in live bivalve molluscs (ISO TS 16649-3).

12. The workshop noted that following technical evaluation by the CRL, the CRL considered that the validation was scientifically satisfactory and therefore suitable for use in French Official Control laboratory testing under the supervision of NRL France.

13. Further to the above the CRL noted that the procedure for recognition of validated alternative methods was not clear. DG SANCO would clarify the procedure for formal acceptance of alternative methods for use in OC testing and report back to the CRL. The CRL would subsequently inform the network.

14. NRL France stated that in the absence of an internationally recognised standard, to ensure appropriate application and quality controls, the protocols required for application of the impedance method using Bactrac 4300 would be placed in the public domain.

Microbiological methods – sanitary surveys

15. The workshop noted that there were large disparities in the approach to conducting sanitary surveys across Member States. Some MS reported no activity whereas other MS had instigated a more systematic approach. All MS prioritised sanitary surveys in new areas, many MS reported no activity since no new areas had been established (in line with Commission guidance). The Commission elaborated that reclassified areas also required a sanitary survey. Where sanitary surveys were being performed MS were following, fully or in part, the guidance contained with the GPG.

16. The CRL agreed to summarise the information provided by NRLs with respect to application of sanitary surveys in a short report and circulate to NRLs and to DG SANCO and place on the CRL website.

17. The network noted that the absence of systematic sanitary surveys in some MS resulted in sampling plans for monitoring programmes that were not based on scientific principles.

18. The CRL agreed that, subject to there being sufficient interest, a further training course on the application of sanitary surveys would be held in the autumn of 2009. NRLs agreed to consult with the responsible bodies (e.g. the Competent Authority) to identify appropriate participants.

19. NRLs welcomed the information from DG ENVIRONMENT that, following the repeal of the Shellfish Waters Directive (2006/113/EC), the Water Framework Directive would continue to ensure the same level of environmental protection for all designated shellfish harvesting areas. NRLs asked the CRL to clarify with the Commission whether the mechanism for this would be through the continuation the existing standard or an alternative approach.

Microbiological methods – Proficiency testing for statutory determinands

20. NRLs supported the need for a harmonised approach across the network for assessment and follow-up activity with respect to supervision of Official Control laboratory performance in proficiency testing. Amendments agreed to the CRL proposal were accepted by workshop. On completion the document would be placed on the CRL website.

21. NRLs agreed to continue to participate in the CRL/HPA EQA for *E.coli* and *Salmonella* spp as a mandatory requirement. It was agreed that laboratories should participate in sufficient distributions (currently 2 samples tri-annually) to enable full assessments of laboratory performance.

22. Further to the above NRLs also agreed the continuing need for proficiency testing using matrix samples as a mandatory requirement. CRL agreed to organise a further distribution of whole bivalve shellfish for enumeration of *E. coli* and detection of *Salmonella* spp.

23. Further to the above, participation in the whole bivalve shellfish proficiency test would be open to Official Control laboratories nominated by NRLs. The CRL would need to recover the costs for Official Control laboratory participation.

Viruses

24. NRLs noted the value of the CRL norovirus and hepatitis A proficiency-testing scheme and requested that a further lenticule distribution should be offered in 2009/10. Samples would be available to non-NRL laboratories on a cost recovery basis.

25. In addition, NRLs requested that the CRL organise a parallel distribution of contaminated bivalve shellfish (norovirus and hepatitis A) to challenge aspects of the virus methodology that are not currently examined using laboratory constructed materials. This would be subject to budgetary agreement with the Commission for sample transport.

26. In the absence of certified reference materials the CRL agreed to continue make available norovirus and hepatitis A reference material in lenticule format with supporting CRL derived reference values and strain characterisation (for norovirus).

27. NRLs supported the work of CEN/TC275/WG6/TAG4 'Viruses in food' on the development of a standard method for detection of norovirus and hepatitis A virus in bivalve molluscs. Subject to laboratories agreeing to adhere to the technical protocols and to make data available to TAG4, then standard operating procedures would be made available to interested laboratories for research purposes only.

28. NRL France agreed to provide the draft documents prepared by the Codex committee on food hygiene describing proposals for the reduction viruses in all foodstuffs to the CRL for circulation to the network. NRL observations with respect to the proposal must be received by the 23rd May to respect the deadline for comment to the Codex committee.

Vibrios

29. NRLs noted the value of the dedicated vibrio session and expressed sincere thanks to the EU National and International experts for their contributions to the workshop.

30. NRLs reported that officially recorded illness associated with consumption of bivalve molluscs appeared low throughout the EU and that monitoring of either Community products or imports

was not systematically applied. Some MS had risk based plans for monitoring imports. A variety of methods were in use for vibrio testing in MS. The CRL agreed to compile a dossier of the information supplied by NRLs, distribute to NRLs for comment and on completion place on the CRL website (NRL restricted section).

31. It was acknowledged that the outcomes of research surveillance studies carried by some NRLs provided useful information. However, the lack of harmonised methods in the network made it difficult to compare these data.

32. NRLs noted the results of the vibrio CRL proficiency testing and acknowledged the apparent variability in participants' performance. It was recommended that details on methodologies used by participants' should be sought and included in the final report. The CRL would check the designation of strains of *V. parahaemolyticus* used in the ring trial with respect to presence of *trh1* and *trh2* genes and include this information in the final report.

33. NRLs requested a further proficiency testing distribution for enumeration of total and pathogenic for *V. parahaemolyticus.* Expressions of interest would be invited amongst the network.

34. The workshop noted that although vibrio risk currently appeared to be low in EU MS, international experience demonstrated that outbreaks can occur unexpectedly. It was acknowledged that imports into the EU from endemic areas also presented a risk. Thus it was important for laboratories to be well prepared.
35. Further to the above NRLs considered that it was necessary to further develop and improve fit-for-purpose vibrio methods for use by the network. Critical aspects included method sensitivity, determination of putative pathogenicity principles, and the requirement for quantitation. From data presented it was apparent that molecular methods may offer significant advantages in this respect.

36. Further to the above the network agreed to establish a working group to make recommendations on fit-for-purpose methods for vibrio determination and their potential application. NRL representatives agreed to nominated working group members with appropriate expertise to the CRL by the end of June 2009.

Next meeting

37. Provisionally it was agreed that the next workshop of NRLs would be hosted by NRL Italy, Ancona. The provisional date of the next workshop would be between the 18th - 20th May 2010.

Annex III – Agenda of the 8th workshop of NRLs for bacteriological and viral contamination of bivalve molluscs, 2009.

AGENDA

8th Workshop of Microbiological NRLs, 12-14 May 2009

Venue: Tryp Bosque Hotel
Camilo Jose Cela 5
07014
Palma de Majorca
Balearic Islands
Spain

Tel: 0034 971734445
Fax: 0034 971733444

Enquiries: Prior to the workshop enquiries should be directed to Rachel Rangdale:

CRL Co-ordinator
Cefas Weymouth Laboratory,
Barrack Road,
The Nothe,
Weymouth, Dorset, DT4 8UB, UK
Telephone : +44 (0) 1305 206600
Direct line : +44 (0) 1305 206707
Fax : +44 (0) 1305 206601
E-mail : mailto:rachel.rangdale@cefas.co.uk

Day 1 - Tuesday 12 May 9:00 – 17:00

1 Welcome meeting

- **1.1** Welcome and introductions.
- **1.2** Domestic arrangements including reclaim of expenses (papers WS08/01, WS08/02).
- **1.3** Actions arising from the 7th workshop 2008 (paper WS08/03).
- **1.4** Agreement of the agenda (paper WS08/04).

2 Microbiological monitoring- statutory determinands

- **2.1** Update on progress of the Microbiological monitoring of bivalve mollusc harvesting areas – guide to good practice: technical application (DG SANCO).
- **2.2** Codex Standard for live and raw bivalve molluscs - Codex Stan. 292-2008 (CRL) (paper WS08/06, WS08/07).
- **2.3** Analytical tolerance in 10% of Class B LBM (Commission Regulation (EC) 1021/2008) (CRL) (paper WS08/08).
- **2.4** Validation of the Impedance technique for enumeration of *E.coli* in live bivalve molluscs - Interpretation of impedance curves and use condition in routine (Martial Catherine, NRL France).
- **2.5** Update on the progress of the validation of ISO 16649-2 (TBX method) (NRL, The Netherlands).
- **2.6** Update on application of Water Framework Directive (2006/113/EC) (CRL) (paper WS08/09).

3 Microbiological monitoring- sanitary surveys

3.1 Round table contributions from laboratories on activities relating to application of sanitary surveys across the network (All NRLs with LBM production areas).

3.2 Report on the first International workshop of Sanitary Surveys (CRL) (paper WS08/10).

3.3 Update on requirements for sanitary surveys under Commission Regulation (EC) No.854/2004 (DG SANCO).

Lunch

4 Proficiency testing (PT) programmes

4.1 NRLs participation and performance in the CRL/HPA Shellfish EQA for *E. coli* and*Salmonella* (CRL) (paper WS08/11-draft).

4.2 NRLs participation and performance in the CRL whole animal distribution for *E. coli* and*Salmonella* (CRL) (paper WS08/12).

4.3 Discussion on PT performance assessment and follow-up activities (CRL) (paper WS08/13).

5 Viruses

5.1 Update on the progress of virus standard at CEN/TC275/WG6 and virus validation (M/381) (CRL).

5.2 Report on NRLs participation in RT25 norovirus and hepatitis A PT (CRL) (paperWS08/14).

5.3 Evaluation of NoV contamination of mussels from Campania region using two Real TimePCR methods. (NRL Italy).

5.4 First meeting of the Codex working group viruses on the development of a code of hygienic practice for control of viruses in food. (NRL France).

5.5 Outbreaks, reporting and the use of virus test results (contributions from NRLs).

5.6 Other contributions of virus issues from the network.

Day 2 - Wednesday 13 May 9:00 - 17:30

6 Vibrio session- Introduction

6.1 Introduction – brief overview of human pathogenic spp. and public health significance in Europe (CRL) (paper WS08/15).

6.2 Round table contributions from laboratories on approach to testing and controls in Member States.

7 Vibrio session- methodology and proficiency testing

7.1 Update on progress at CEN/TC275/WG6/TAG3 on *V. parahaemolyticus* methodology development (CRL).

7.2 Report on NRLs participation in RT29 *Vibrio* spp. PT (CRL) (paper WS08/16- draft).

7.3 Series of round robin trials using different PCR methods on in cell cultures and oyster homogenates (Dr Andy De Paola US).

7.4 Development of real-time PCR for *V. parahaemolyticus*. (NRL France).

7.5 Methods to detect *Vibrio* spp. in Sweden (NRL Sweden).

Lunch

8 Surveillance, outbreaks and investigations

8.1 Result of surveillance studies of total and entropathogenic *V. parahaemolyticus* in Slovenia (NRL Slovenia).

8.2 *V. parahaemolyticus* from shellfish and clinical sources in Italy (NRL Italy).

8.3 Epidemiology and biology of the new O3:K6 clone of *V. parahaemolyticus* causing pandemic spread of infection from Asia. (Prof Nishibuchi, Japan).

8.4 *Vibrio* spp. monitoring in The Netherlands (NRL the Netherlands).

8.5 Epidemiology and biology of *V. parahaemolyticus* in seafood in Chile (Prof Espejo, Chile).

8.6 Epidemiology of *Vibrio* diseases in Spain and Peru from an oceanographic perspective (Jaime Martinez-Urtaza, Spain).

8.7 Importance of bivalves as a reservoir and the rote of international spread of the pandemic clone of *V. parahaemolyticus* (Prof Nishibuchi, Japan).

8.8 *V. vulnificus* and *V.parahaemolyticus* control plans in the U.S. – Rationale, implementation and efficacy (Dr Andy De Paola US).

8.9 General discussion.

Day 3 - Thursday 14 Day 9.00 - 12.00 (closed meeting)

9 Agreement of Workshop resolutions

10 Any other business

11 Date and venue for next meeting

Meeting close

Annex IV – Sanitary survey – training workshop 2009.

Sanitary surveys- training workshop 2009,
Weymouth and Portland Sailing Academy
Weymouth
http://www.wpnsa.org.uk/index.htm

Tuesday 13th- Friday 16th October 2009

	Day 1: Tuesday 13th October 9:15-18:00	**Speakers/facilitators**
1.	Overview of sanitary survey requirements and aims of the course (9:15-9:45)	Ron Lee, CRL
2	Identification of pollution sources (09:45-10:30)	Simon Kershaw, CRL
3	Data handling and management, including use of GIS (10:30-11:15)	Owen Morgan, CRL
	Coffee	
4	Movement of pollutants in the vicinity of bivalve molluscs production areas (11:30 - 12:15)	John Aldridge, Cefas
5	Analysis of historical microbiological data (spatial, temporal, environmental factors) (12:15:12:45)	Carlos Campos CRL
	Lunch (12:45-13:30)	
6	Overall assessment and establishing sampling plans (13:30-14:15)	Isabelle Amouroux, IFREMER, France
7	Practical demonstration of the use of GIS (14:15-14:45)	Owen Morgan, CRL
8	Desk study (14:45 – 18:00) - Overview of a local bivalve shellfish harvesting area. Working in small groups delegates will identify data requirements, undertake a desk based sanitary survey as outlined in the Good Practice Guide, and to construct a sampling plan with scientific justification for presentation to the group.	Simon Kershaw, CRL

Workshop programme cont.

	Day 2: Wednesday 14th October 9:30-18:00	**Speakers/facilitators**
9	Desk study (9:30 – 15:30) - continuation of the desk study exercise Presentation of outcomes to group (15:45 -17:00)	Carlos Campos Michelle Price-Hayward Ron Lee Simon Kershaw Owen Morgan
10	Overview of the shoreline survey work (17.00-17.30)	Carlos Campos, CRL
11	Use of fieldwork equipment, including the use of GPS (17.30-18.00)	Michelle-Price-Hayward/Owen Morgan, CRL
	Day 3: Thursday 15th October 7:45-18:00	**Speakers/facilitators**
12	Shoreline survey (7:45-18:00) - delegates will be taken to a local bivalve shellfishery, practical training on how to conduct a shoreline survey including offshore surveying will be provided.	Carlos Campos Michelle Price-Hayward Ron Lee Simon Kershaw Owen Morgan John Aldridge
	Day 4 Friday 16th October 9:30-15:00	**Speakers/facilitators**
13	Construction of report/sampling plans (9:30-13:00)- Delegates will combine outputs from the desk study and shoreline survey and produce completed sanitary survey reports and sampling plans for presentation to the group	Carlos Campos Michelle Price-Hayward Ron Lee Simon Kershaw Owen Morgan
	Lunch (13:00-13:30)	
14	Report back by groups (13:30 – 15:00)	Ron Lee, CRL
	Workshop close 15:00	

Annex V - Summary of participation amongst NRLs and others in CRL organised proficiency testing

CRL ring trial reference number	Ring trial description	Austria	Belgium and Luxembourg	Bulgaria	Cyprus	Czech Republic	Denmark	Estonia	Finland	France	Germany	Greece	Hungary	Ireland	Italy	Latvia	Lithuania	Malta	Netherlands	Poland	Portugal	Romania	Slovakia	Slovenia	Spain	Sweden	United Kingdom	Croatia	Turkey	Iceland	Norway	United States	New Zealand	South Korea	Chile	Hong Kong	Canada
RT 27	Norovirus/Hepatitis A 2009	✓	✓	x	x	x	✓	x	x	✓	✓	x	x	✓	✓	✓	x	x	✓	✓	✓	x	✓	✓	✓	✓	✓	x	x	✓	✓	x	✓	✓	✓	x	x
RT 29	*V. parahaemolyticus* 2009	✓	x	x	x	x	x	x	x	x	✓	✓	x	x	✓	✓	x	x	✓	✓	x	x	x	✓	x	x	✓	✓	x	✓	x	x	✓	x	✓	x	x
RT 31	*E.coli/Salmonella* whole animal 2009	✓	x	x	x	x	x	x	x	✓	✓	✓	✓	✓	✓	✓	x	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x	x	✓	x	x	x	x	x	x
RT 36	*E. coli/Salmonella* EQA 2009	✓	x	x	x	x	✓	x	✓	✓	✓	x	x	✓	✓	x	✓	x	✓	✓	✓	✓	x	✓	✓	✓	✓	✓	x	x	✓	x	x	x	x	x	x

Community Reference Laboratory (CRL)

Proficiency Testing Schemes

Enumeration of *Escherichia coli* and the detection of *Salmonella* spp. in Pacific oysters (*Crassostrea gigas*)

CRL ring trial reference number: RT 31

Sample numbers: RT31A and RT31B

Contents	Page number
Samples RT31A and RT31B	2
Results	2
General comments	5
Results chart RT31B	7
Appendices	8
Health and safety	21

This scheme is intended to provide proficiency testing samples for laboratories undertaking examination of live bivalve molluscs from production areas in accordance with Regulation (EC) No. 854/2004 and from throughout the production chain in accordance with Regulation (EC) No. 2073/2005.

The scheme is organised by the Community Reference laboratory (CRL) for monitoring bacteriological and viral contamination of bivalve molluscs. The NRL is designated by the European Commission in accordance with Regulation (EC) No. 882/2004. The scheme is intended to compliment the CRL/HPA Shellfish Scheme (www.hpa.org.uk) through examination of aspects of the methods not covered under the Shellfish Scheme (initial sample preparation and preparation of initial dilutions).

The EU stipulated reference method for enumeration of *E. coli* in live bivalve molluscs in ISO TS 16649-3, Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Anon 2005). The EU stipulated reference method for detection of *Salmonella* spp. in live bivalve molluscs is ISO 6579, Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. (Anon 2002).

A scoring system is used to help assess participants' performance. Details of this system are included as Appendix I of this report. The purpose of scoring is to help the CRL, member state NRLs and other participating laboratories identify incorrect or outlying results. Further information on the use of scoring in proficiency testing and on recommended procedures for following up poor performance is included as Appendix II, can be accessed via the CRL website (www.crlcefas.co.uk) or obtained by contacting the CRL. The European Commission has produced a protocol for management of underperformance in comparative testing and/or lack of collaboration of NRLs with CRLs activities. This is reproduced as Appendix II of this report or can be obtained by contacting the CRL.

If you are experiencing problems with any aspects of these distributions please contact the CRL (contact details below), or alternately refer to the troubleshooting guide included as Appendix IV of this report.

Further advice on microbiological testing of bivalve mollluscan shellfish can be obtained via the NRL website (www.nrlcefas.org)

Due to the nature of this scheme repeat samples are not available.

Distribution date:	**12th October 2009**
Report date:	**22nd December 2009**
Report compiled by:	**Louise Stockley** **Rachel Rangdale**
Authorisation by:	**Rachel Rangdale**

Samples

Sample preparation

Two batches each consisting of 800 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area classified according to Regulation (EC) 854/2004 as class B.

Note: Repeat samples are not available for this scheme

Sample RT31A

One batch of oysters was bio-accumulated with *Salmonella* Bristol (NCTC 9853) according to NRL standard procedures. Oysters were stomached and pooled to form one homogeneous sample. Each sample of RT31A comprised a single aliquot of 100ml of stomached shellfish flesh and intravalvular fluid.

Sample RT31B

Each sample of RT31B comprised 15 randomly selected whole *C. gigas* directly from the harvesting area.

Sample distribution and examination

Samples were distributed refrigerated on 12th October 2009 to thirty-three participating laboratories. Participants were required to analyse the material in duplicate immediately on receipt using their routine laboratory procedures. Supplementary advice on sample acceptance, receipt and processing is available as Appendix V or via the CRL website (HUwww.crlcefas.orgUH)

.

Sample temperature

Temperature recorders (Thermotrack, Progress Plus) were included in each consignment. Participants were required to record the internal air and sample temperature on arrival and to return the recorder.

Results

Reference results

Sample RT31A: Twelve randomly selected sub-samples of RT31A were analysed for *Salmonella* spp. using CRL SOP No 1176 http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_SALMONELLA_17_11_07.pdf applied in a 3 x 3 MPN format (Table 1).

Note: Regulation (EC) No. 2073/2005 requires presence/absence testing for *Salmonella* spp. in live bivalve molluscs. Quantitative data is provided for information only.

Table 1: Reference results – Sample RT31A

Sample description	*Salmonella spp.*	No. of replicates giving positive results	Mean MPN *Salmonella* spp. per g
RT31A	*Salmonella* spp. detected in 25g	12	3.6×10^5

Sample RT31B: Twelve randomly selected sub-samples of RT31B were analysed for *E. coli* using CRL SOP No. 1175 http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_E_coli_04_04_08.pdf (Table 2).

Table 2: Reference results – Sample RT31B

Sample description	*E. coli* MPN/100g			
	Range	Median	GM	Median $\pm 3*SD_T$
RT31B	$<2.0 \times 10^1$ - 3.3×10^2	2.3×10^2	1.8×10^2	3.8×10^1 - 1.4×10^3

GM- geometric mean, SD_T – theoretical standard deviation

Participants' results

Performance assessment was performed according to the procedures described in the CRL/HPA EQA shellfish scheme for a single distribution, with minor modifications (Appendix I).

Sample RT31A

Participants' results and scores allocated for sample RT31A are shown in Table 3.

Summary statistics – Sample RT31A

Total participants returning results	33
Participants reporting expected result	33

Sample RT31B

Participants' results and scores allocated for sample RT31B are shown in Tables 3, 4 and Figure 1.

Note: The median and upper and lower limits (±3 SD and ±5 SD) were calculated from participants' results. SD calculations were based on the inherent variability of the 5 x 3 MPN method (0.26 $\log_{10}$). Reference values were excluded from calculation of participants' median.

Summary statistics – Sample RT31B

Total participants reporting duplicate results for *E. coli* MPN	33
Number of outlying results	1
Participants reporting MPN results within the expected range[1]	30
Participants reporting MPN results outside the expected range for one replicate	2
Participants reporting MPN results outside the expected range for both replicate	1
Participants reporting MPN results inconsistent with ISO 7128 (Anon 2007a)[2]	3

[1]expected range = participants' median ± theoretical 3SD

[2] on this occasion points were not deducted from participants returning results inconsistent with ISO 7128, laboratories were reminded that 5 x 3 MPN tables from ISO 7128 or those provided by the CRL should be used for MPN determination

Table 3: Participants results and allocated scores

Lab ID	RT31A *Salmonella* spp. in 25g		RT31B *E. coli* MPN/100g		
	Result	Score	Replicate 1	Replicate 2	Score
3*	Present	2	20	50	12
7*	Present	2	<20	<20	12
9*	Present	2	<20	20	12
10*	Present	2	130	130	6
13*	Present	2	50	20	12
19*	Present	2	1300	490	2
21	Present	2	40	<20	12
22*	Present	2	70	170	9
27*	Present	2	<20	<20	12
30	Present	2	<20	20	12
32*	Present	2	20	<20	12
33*	Present	2	<20	<20	12
35*	Present	2	80	<20	12
39*	Present	2	20	20	12
41*	Present	2	45	20	12
43*	Present	2	20	20	12
44*	Present	2	<20	<20	12
47*	Present	2	<20	<20	12
54	Present	2	40	50	12
56	Present	2	<20	20	12
58	Present	2	20	80	12
68*	Present	2	<20	<20	12
82	Present	2	<20	<20	12
86	Present	2	<20	50	12
90	Present	2	20	80	12
106	Present	2	<20	<20	12
111	Present	2	80	20	12
119	Present	2	20	20	12
126	Present	2	80	50	12
132	Present	2	<20	<20	12
144	Present	2	90	40	12
147*	Present	2	20	20	12
149	Present	2	<20	20	12

* Designated NRL's

Table 4: Participants results RT31B

	E. coli MPN/100g			
	Range	Median	GM	Median±3*SD_T
Participants results	$<2.0 \times 10^1$-1.3×10^3	2.0×10^1	2.4×10^1	$3.0 – 1.2 \times 10^2$

GM- geometric mean, SD_T – theoretical standard deviation

General comments
Thirty-three laboratories (18 NRL and 15 other laboratories) returned results for this distribution. Sixty-one percent of samples arrived within 24 hr of dispatch. Eighteen laboratories analysed the samples on the day of arrival. Of the remaining 15 laboratories, 11 analysed on the following day (i.e. within 48-72 hr of dispatch). One laboratory analysed the sample 5 days after receipt into the laboratory. The maximum internal temperature of samples recorded by participants on arrival did not exceed 10°C. Temperature loggers showed an in transit temperature, range of 2.5 - 7.5°C, temperature data for participants are given in Appendix VI. Thirteen samples failed to reach their destination laboratory within the 24-hours recommended in the microbiological monitoring of bivalve mollusc harvesting areas, a guide to good practice: technical application (Anon 2007b). Two samples arrived outside of 48 hours. Notwithstanding this laboratories analysed the samples on arrival, the results did not appear to have been affected by the extended transport time.

Sample RT31A
All laboratories returned expected results for RT31A, this is however unsurprising as levels of *Salmonella* spp. in the bio-accumulated oysters were exceptionally high. It is noted that levels <100cfu/25g would be required to fully challenge the methodology.

Eighteen laboratories used the EU specified reference method for detection of *Salmonella* spp (ISO 6579). Three laboratories referenced NMKL 71: *Salmonella* detection in foods. Two laboratories referenced Vidas, Elisa method and BAX (PCR method) to detect for *Salmonella* analysis. **Laboratories are reminded that for official control testing of live bivalve molluscs for *Salmonella* spp the EU reference method is ISO 6579, Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.** (Anon 2002). Alternative methods should be validated against this reference according to ISO 16140 Microbiology of food and animal feeding stuffs – protocol for validation of alternative methods (Anon 2005) before use in official control testing.

Sample RT31B
Thirty laboratories returned both replicate *E. coli* MPN/100g results between ±3 SD of the participants' median for RT31B. Laboratory 10 returned two replicate results above +3 SD but below +5 SD of the participants' median. Laboratory 22 returned one replicate result between +3 and +5 SD of the participants' median. Laboratory 19 returned both replicates outside 5 SD of the participants' median. It is recommended that laboratory 19 critically examine its procedures for analysis of bivalve shellfish for *E. coli* and if necessary consult the CRL for assistance.

Laboratories 21, 41 and 144 reported one or both MPN value(s) that were not consistent with 5 x 3 MPN tables in ISO 7218, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations (Anon 2007) or 5 x 3 MPN tables derived from this standard and previously supplied to NRLs by the CRL. Laboratories are reminded that that 5 x 3 MPN tables from ISO 7128 or those provided by the CRL should be used for MPN determination (Appendix VII). On this occasion points were not deducted from participants returning inconsistent results in future PT distributions points will be deducted for failure to use specified tables and laboratories should update their procedures accordingly.

Twenty-two laboratories cited ISO TS 16649-3 (Anon 2005) or a derivative of this as their laboratory method for enumeration of *E. coli* in sample RT31B. Laboratories are reminded that 5 x 3 MPN tables in Donovan *et al* (1998) and those contained in ISO 7251 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique, differ slightly from those contained in ISO 7218. **Laboratories are reminded that for enumeration of *E. coli* in live bivalve molluscs for official control testing using ISO 16649-3 5 x 3 MPN Tables in ISO 7218 or those provided by the CRL should be used.**

References
Anon 2007a ISO 7218:2007 Microbiology of food and animal feeding stuffs - General recommendations and guidance for microbiological examinations. Geneva, Switzerland.

Anon 2007b. Microbiological monitoring of bivalve mollusc harvesting areas, a guide to good practice: technical application. CRL publication, issue 3, February 2007.

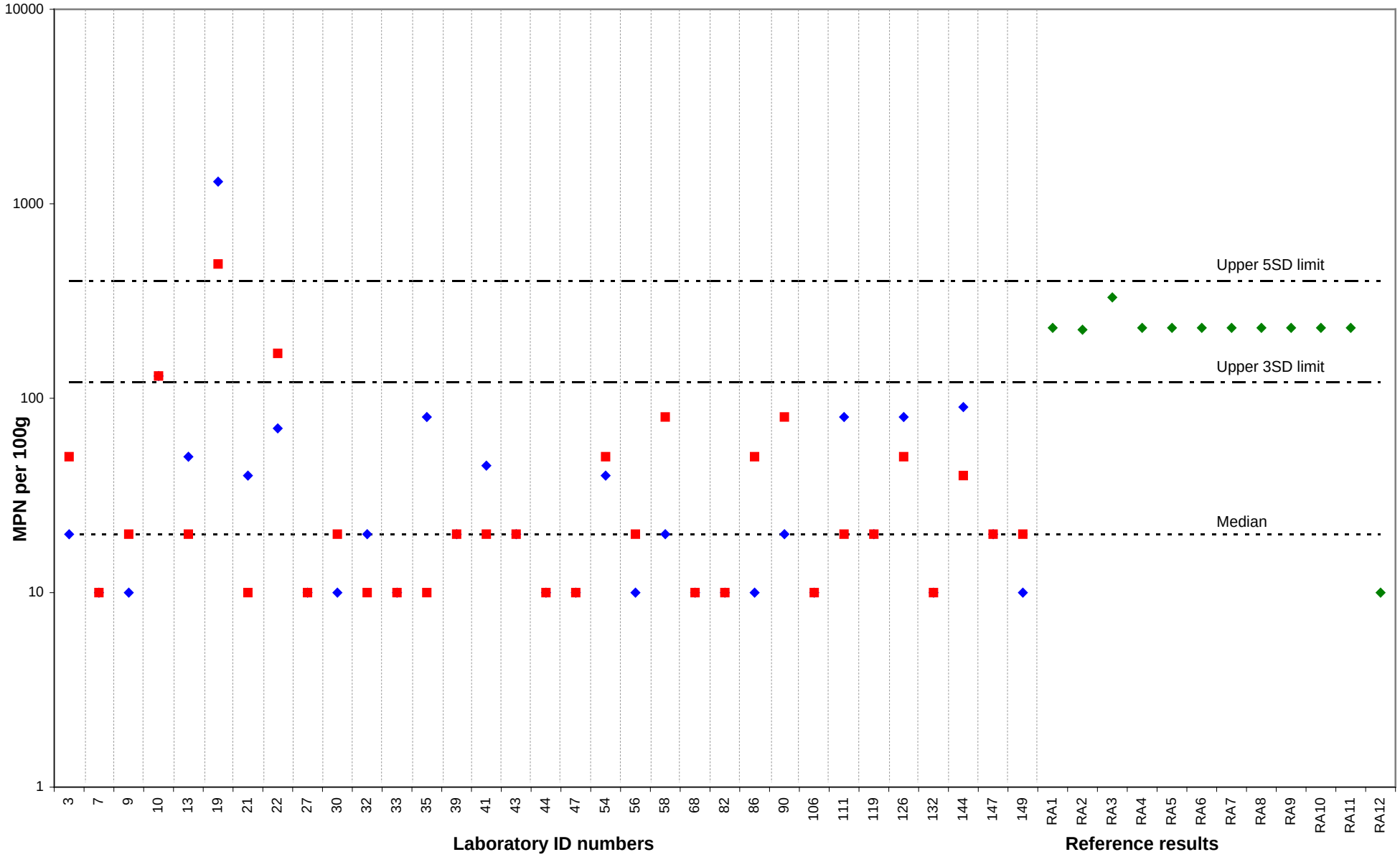
Anon 2005a ISO TS 16649-3:2005. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Geneva, Switzerland.
Anon 2005a ISO 16140:2005. Microbiology of food and animal feeding stuffs - protocol for validation of alternative methods. Geneva, Switzerland.

Anon. 2002. ISO 6579:2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva, Switzerland.

Donovan TJ, Gallacher S, Andrews NJ, Greenwood MH, Graham J, Russel JE, Roberts D, Lee R. (1998). 'Modification of the standard method used in the united kingdom for counting *Escherichia coli* in live bivalve molluscs'. Communicable disease and public health 1: 188-96.

Cefas

Results chart - Sample RT31B



Appendix I:

***E.coli* MPN scoring**

Result	Score allocated
Return of results	2
All replicate MPN results within the expected range	10
Or	
One replicate MPN result reported is outside the expected range and falls between the median ±3SD and median ±5SD value	7
Or	
Both replicate MPN results are outside the expected range and fall between the median ±3SD and median ±5SD value	4
Or	
One replicate MPN result reported is outside the median ±5SD value	5
Or	
Both replicate MPN results reported is outside the median ±5SD value	0
Or	
Single MPN result reported only	5
Or	
Tube combination inconsistent with MPN reported (one replicate)	7[1]
Or	
Tube combination inconsistent with MPN reported (both replicates)	5[1]
Or	
Sample not examined or results returned late - no explanation received	0
Or	
High censored result (e.g. MPN = >18000 per 100g)	Score not allocated

[1]on this occasion points were not deducted from participants returning results inconsistent with ISO 7128, laboratories were reminded that 5 x 3 MPN tables from ISO 7128 or those provided by the NRL should be used for MPN determination

***Salmonella* spp scoring**

Result	Score allocated
Fully correct results	2
Misleading result, e.g. failure to isolate *Salmonella*	0

Appendix II

Protocol for management of underperformance in comparative testing and/or lack of collaboration of National Reference Laboratories (NRLs) with Community reference laboratories (CRLs) activities

According to article 32 of Regulation (EC) 882/2004[1], Community Reference Laboratories (CRLs) shall be responsible for coordinating application by the NRLs of analytical methods, in particular by organising comparative testing and by ensuring an appropriate follow-up of such comparative testing.

Article 33 of the Regulation establishes that NRLs shall collaborate with the CRLs in their area of competence.

The NRLs are a key tool for the proper implementation of official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, therefore their performance is of outmost importance.

Appropriate actions must be taken if the results of comparative tests reveal underperformance or if NRLs fail to collaborate properly with the corresponding designated CRL.

The following two-step protocol is suggested in case of

(a) underperformance (i.e. failure in proficiency test)

(b) lack of collaboration by the NRLs with the CRL

Phase 1

(a) Underperformance (i.e. failure in proficiency test)

- CRL should contact the NRL and provide assistance trying to identify the origin of the bad result. On the spot visits and training could be foreseen if necessary.

- Repetition of the comparative test if feasible (e.g. within 3 months) and close assessment of the results by the CRL

Confidentiality should be kept during this phase in order to ensure good co-operation from the NRL. The results of the proficiency test and the codes of the laboratories are included in the report transmitted to the Commission. Apart from that there is no need to further involvement of the Commission until the results of the following comparative test are available and re-assessed.

[1] OJ L 165, 30.4.2004, p. 1, corrected by OJ L 191, 28.5.2004, p. 1. Regulation as last amended by Commission Regulation (EC) No 776/2006 (OJ L 136, 24.5.2006, p. 3)

(b) Lack of collaboration by the NRLs with the CRL:

- CRL should contact the NRL if lack of collaboration with CRL activities. CRL should ask the NRL for the reasons of no participation to a proficiency test or a workshop. The justification provided by the NRL should be included in the report submitted to the Commission

Phase 2

(c) Underperformance (i.e. fail in proficiency test)

- If the results of the following comparative test still reveal underperformance of the NRL or the collaboration of the NRL is not adequate, the Commission shall be informed officially by the CRL including a report of the main findings and corrective actions to improve the situation.

- The Commission shall inform the competent authority and require that appropriate actions are taken.

(d) Continuous lack of collaboration by the NRLs with the CRL:

- In case of repetitiveness of the lack of response of the NRL, the Commission shall be informed officially by the CRL and the Commission shall inform the competent authority and require that appropriate actions are taken.

Appendix III

Guidance on performance assessment in proficiency testing and follow-up activities
Version 1.1 December 2009

Introduction
Article 33 of Regulation (EC) No 882/2004 of the European Parliament and of the Council on Official Controls performed to ensure the verification of compliance with feed and food law (Anon 2004) sets out the remit of European National Reference laboratories (NRLs). This article specifies that where appropriate, NRLs should organise comparative tests, also known as proficiency testing (PT), between official laboratories and ensure an appropriate follow-up of such comparative testing.

All laboratories undertaking official controls on live bivalve molluscs should participate in a relevant PT scheme organised by their NRL or another designated programme (e.g. those organised by the CRL). Proficiency testing enables both an independent assessment of laboratory performance and comparative performance assessments with other participants. The frequency of such participation should be at least biannual to enable identification of poor performance over a realistic timescale. Laboratory performance should be monitored by the NRL on a regular basis. Poor performance should be investigated and reasons for failures identified. Laboratories that continually or persistently fail in proficiency tests may be suspended from official control testing by the relevant authorities.

Frequently PT schemes utilise statistical approaches to assess participant's performance and assign acceptability criteria. The following document describes an approach to assessing performance in comparative testing based upon allocation of numerical scores. Examples of follow-up procedures and suggested courses of action in the event of continual or persistence poor performance are provided.

The use of scoring
Allocation of scores in PT schemes enables measurement of performance based on empirical data. The advantages of the use of scoring in proficiency testing are listed below.

- Scoring systems are used to help assess participants' results in PT schemes. Allocation of scores helps participants', and other entities (CRL, NRL, Accreditation bodies), assess their performance.
- Scores can be used to assess performance in a single distribution (or sample) and to monitor ongoing performance over time with assessments on cumulative scores over a specified timeframe or number of distributions.
- Scores help scheme organisers recognise those participants' who experience problems and thus enable provision of additional help, advice and support.
- Scores are usually allocated following statistical analysis of participants' results. It is important that scoring procedures are reviewed frequently to ensure continued fitness for purpose.

Monitoring of laboratory performance
Laboratory performance should be monitored frequently and according to a defined schedule. Where poor performance is noted certain procedures should be instigated. When scoring systems are utilised failures may be identified by participants' scores that fall outside of defined performance criteria. Such occurrences should trigger follow-up activities by NRLs, all PT failures should be examined by the NRL. Follow-up procedures should be fit for purpose and regularly reviewed.

In the first instance it is recommended that the laboratory experiencing a failure in a proficiency test should be contacted and reasons for failure identified. This will enable the laboratory to conduct an investigation under their quality procedures into the nature of the failure and if available repeat the test.

The NRL should undertake proactive checks covering OCL performance in PT on at least an annual basis

Example follow-up procedures
Follow-up procedures can include:

- Examination of methodology in use, through for example, scrutiny of the laboratories standard operating procedures and result interpretation/reporting protocols.
- For culture based methods in microbiology e.g. ISO TS 16649-3 and EN/ISO 6579, quality control information of

media should be scrutinized to ensure that media is performing adequately.

- Equipment records for equipment used in the procedures (e.g. incubators, measuring instruments, refrigerators) should be checked to ensure appropriate calibration, maintenance and performance.
- Staff training records should be examined to ensure that staff are adequately trained; familiar with procedures and that ongoing checks of staff competence are in place.
- Clerical procedures should be scrutinized to ensure that sample receipt, sample labeling, laboratory numbering and supporting clerical procedures are in place. It is worthy of note that frequently failures in proficiency testing can stem from failure to return results within a specified time frame. Laboratory systems should be in place to ensure that results are reported accurately and on time.
- Accreditation records should be checked to ensure that staff adhere the laboratory quality policy at all times.
- The use of, type and relevance of internal quality controls should be examined.
- Laboratory quality procedures for reacting to internal/external quality control failures.
- Onsite observation of practices in the poorly performing laboratories.

Corrective Actions

If a laboratory continues to fail in a proficiency test (or series of tests), or fails to provide adequate justification for the responsible authorities should be notified.

Continued failure in PT may result in the formal removal of the laboratory from official control testing.

References

Anon 2004. European Communities 2004. Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Off. J. Eur. Communities L 165, 30.4.04 : 1-141.

Appendix IV:

Troubleshooting advice

1. Methods – Ensure that the method used is appropriate for the examination of the sample.

    a. Ensure that any dilutions have been calculated correctly.
    b. Ensure that MPN tables (if used) are interpreted correctly.

2. Culture Medium - Check the quality control data for media to ensure that they are within specifications and performing adequately.

3. Equipment - Check that the equipment used for the procedures (incubators, refrigerators, measuring instruments) are calibrated and performing adequately.

4. Staff Training - Check that the staff performing the tests are fully trained and familiar with all the procedural steps.

5. Clerical Procedures - Check that the sample labeling, laboratory numbering and clerical procedures are adequate have you procedures for ensuring that test results are reported accurately and on time.

6. Accreditation- Check that quality procedures are documented and adhered to at all times.

7. Internal quality controls (IQC) – Ensure that adequate controls are in place and that and documentation for dealing with IQC failures is appropriate.

Further advice can be obtained from the NRL on request.

Appendix V
Community Reference Laboratory (CRL) Proficiency Testing Schemes
Instruction sheet for shellfish samples

Supplementary information for whole live bivalve molluscan shellfish proficiency testing schemes

Samples:

- Individual samples in this proficiency testing (PT) scheme each comprise a single species of live bivalve molluscs.
- Commission Regulation EC (No) 2073/2005 on microbiological criteria stipulates the use of a pooled sample of a minimum number of 10 individual animals for enumeration of *Escherichia coli* in live bivalve molluscs. This is to reduce the potential for individual animal-to-animal variation in microbiological content to bias the sample result.
- Samples provided as part of this PT scheme comprise at least 10 animals in the case of smaller species additional animals are provided to ensure that sufficient flesh and intravalvular fluid is provided for the analyses. The PT sample should comprise at least the following numbers of individuals:

Oysters (*Crassostrea gigas* and *Ostrea edulis*)	12 - 18
Mussels (*Mytilus* spp.)	18 - 35
Hard shell clams (*Mercenaria mercenaria*)	12 - 18
Manila clams (*Tapes philippinarum*)	18 - 35
King scallops (*Pecten maximus*)	12 - 18
Queen scallops (*Aequipecten opercularis*)	18 - 35
Cockles (*Cerastoderma edule*)	35 - 55
Razor clams (*Ensis* spp.)	12 - 18
Palourdes (*Tapes decussatus*)	18 – 35

- **Sample temperature**
  - The internal air temperature should be recorded immediately on arrival. The temperature should not exceed 8°C.
  - The sample temperature should be recorded, the sample temperature should be between 1-8°C, if sample temperature exceeds 8°C or is below 1°C **please contact the CRL immediately**. **Samples should not be frozen**.
- Where possible samples should be processed within 24 hours of dispatch, if not processed immediately samples should be stored at 3±2°C.
- Before commencement of analysis shellfish should be examined visually, only animals that are alive according to any of the following criteria should be chosen:
  - Any exposed flesh should react to touch.
  - Shellfish should open and close of their own accord.
  - They should respond to percussion.
  - Tightly closed shellfish.
- Dead or damaged shellfish should be discarded.
- **Samples should not be re-immersed in water.**
- **If the samples are considered unsatisfactory please contact the CRL immediately.**

Reporting:

- Results should be reported on the form supplied by the CRL.
- Both the MPN *E. coli* per 100g and the numerical combination used to determine the MPN should be recorded on the form.
- MPNs should be determined using 5 x 3 MPN tables provided in ISO 7218:2007. ISO 7218:2007 Microbiology of food and animal feeding stuffs - General recommendations and guidance for microbiological examinations.
- For examination for *Salmonella* spp. result should be recorded as presence or absence in 25g.
- The laboratory ID number should be clearly recorded on the form.
- Results should be returned by the date stipulated on the base of the form.

- Results can be faxed, emailed or posted, please ensure to return the form to the nominated named member of the CRL indicated on the base of the form.
- **Results received after the deadline cannot be included in the proficiency testing report.**

Useful references:

- ISO TS 16649-3:2004 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Geneva, Switzerland.
- The CRL standard operating procedure based upon the above standard can be accessed via the CRL website http://www.nrlcefas.org/InformationCentre/docs/NRL_SOP_E_coli_31_03_08.pdf
- Anon. 2002. ISO 6579:2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva, Switzerland.
- The CRL standard operating procedure based upon the above standard can be accessed via the CRL website. http://www.nrlcefas.org/InformationCentre/docs/NRL_SOP_SALMONELLA_17_11_07.pdf
- ISO 7218:2007 Microbiology of food and animal feeding stuffs - General recommendations and guidance for microbiological examinations. Geneva, Switzerland.
- http://www.crlcefas.org/InformationCentre/docs/854_h3oregulation.pdf
- http://www.crlcefas.org/InformationCentre/docs/882_Official_food_feed_controls.pdf
- http://www.crlcefas.org/InformationCentre/docs/20732005_microcriteria.pdf

Useful contacts:

Troubleshooting and microbiological advice: Ms Louise Stockley

Email: louise.stockley@cefas.co.uk
Tel: +44 (0) 1305 206644
Fax: +44 (0) 1305 206601

Dr Rachel Rangdale
Email rachel.rangdale@cefas.co.uk
Tel: +44 (0) 1305 206707
Fax: +44 (0) 1305 206601

General comments, including problems with receipt of samples: Ms Louise Stockley (as above)

CRL Director: Dr David Lees
Email: david.lees@cefas.co.uk
Tel: +44 (0) 1305 206625
Fax: +44 (0) 1305 206601

Appendix VI:
Sample arrival and temperature

Lab ID	Date arrived	Time of arrival	Temp. logger (°C)	Internal air (°C)	Sample (°C)	Storage (°C)
3	13/10/2009	10:00	3 - 6	11.1	7.4	-
7	13/10/2009	14:10	4.5 - 7.5	8.0	7	2 - 4
9	13/10/2009	16:50	-	7.5˚	5	4 - 8
10	13/10/2009	09:30	3 - 6	-	-	-
13	13/10/2009	11:50	3.5 - 6	6.5	5	3.5
19	14/10/2009[1]	14:15	6.5	0.8	0.8	4
21	13/10/2009[1]	14:30	5 - 7.5	11	6.2	-
22	15/10/2009	11:15	6	6.8	9.8	-
27	13/10/2009	12:10	5 - 7	8	7	3±2
30	13/10/2009	13:00	4 - 6	3.4	4.9	2.5
32	13/10/2009	09:30	4 - 6.5	12	10	4
33	13/10/2009	14:15	3 - 5	5.8	4.3	3.5
35	13/10/2009	12:32	5 - 6.5	8.5	3.9	
39	13/10/2009	11:30	4.5 - 6	8	8	42
41	14/10/2009	11:10	5.5 - 7	5.3	5.6	4±2
43	14/10/2009[1]	08:30	2.5 - 6.5	7.0	5	-
44	14/10/2009	15:40	5 - 7.5	7.5	5.1	-
47	14/10/2009	14:45	4.5 - 6	6.4	5.5	1.4
54	14/10/2009	13:00	4.5 - 6	8	5	4
56	13/10/2009	14:30	3.5 - 6.5	6.9	3.4	3
58	13/10/2009	10:30	4 - 6	5.9	4.1	-
68	14/10/2009	12:00	5.5	5	8	4
82	13/10/2009[1]	11:35	-	3.9	7.5	2±2
86	13/10/2009	12:50	<8.5	6.5	5.4	4.4
90	16/10/2009	10:12	4 - 7.5	14.3	10.5	3
106	14/10/2009[1]	13:26		2.0	2	2
111	13/10/2009		4.5 - 7	-	-	2
119	14/10/2009	13:00	4.5 - 6	3	3	4
126	13/10/2009	11:00	2.5 - 6	9.1-0.2-8.9	3.8-0.2-3.6	4.2
132	14/10/2009	12:00	4 - 5.5	5.0	5	4
144	13/10/2009	13:50	4 - 5.5	5.0	4.7	4
147	13/10/3009	15:46	4 - 6	12˚C	-	4
149	14/10/2009	12:05	4.5 - 7	10.8˚C	9.9	2.4

Appendix VII

E.COLI MOST PROBABLE NUMBER (MPN) TABLES

Cefas Standard Operating Procedure – Enumeration of *Escherichia coli* in molluscan shellfish http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_E_coli_04_04_08.pdf Adapted from: ISO 7218:2007.

Table 1: Most probable number of organisms: table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g.

1g	**0.1g**	**0.01g**	MPN/100g	**Category**
0	0	0	<20	
0	1	0	20	2
1	0	0	20	1
1	0	1	40	2
1	1	0	40	1
2	0	0	50	1
2	0	1	70	2
2	1	0	70	1
2	1	1	90	2
2	2	0	90	1
3	0	0	80	1
3	0	1	110	1
3	1	0	110	1
3	1	1	140	2
3	2	0	140	1
3	2	1	170	2
3	3	0	170	2
4	0	0	130	1
4	0	1	170	1
4	1	0	170	1
4	1	1	210	1
4	2	0	220	1
5	0	0	230	1
4	2	1	260	2
4	3	0	270	1
4	3	1	330	2
4	4	0	340	2
5	0	1	310	1
5	1	0	330	1
5	1	1	460	1
5	1	2	630	2
5	2	0	490	1
5	2	1	700	1
5	2	2	940	2
5	3	0	790	1
5	3	1	1100	1
5	3	2	1400	1
5	4	0	1300	1
5	4	1	1700	1
5	4	2	2200	1
5	4	3	2800	2
5	4	4	3500	2
5	5	0	2400	1
5	5	1	3500	1
5	5	2	5400	1
5	5	3	9200	1
5	5	4	16000	1
5	5	5	>18000	

Table 2: Most probable number of organisms: table for multiple tube methods using 5 × 0.1g, 5 × 0.01g, 5 × 0.001 g.

0.1g	0.01g	0.001g	MPN/100g	Category
0	0	0	<200	
0	1	0	200	2
1	0	0	200	1
1	0	1	400	2
1	1	0	400	1
2	0	0	500	1
2	0	1	700	2
2	1	0	700	1
2	1	1	900	2
2	2	0	900	1
3	0	0	800	1
3	0	1	1100	1
3	1	0	1100	1
3	1	1	1400	2
3	2	0	1400	1
3	2	1	1700	2
3	3	0	1700	2
4	0	0	1300	1
4	0	1	1700	1
4	1	0	1700	1
4	1	1	2100	1
4	2	0	2200	1
5	0	0	2300	1
4	2	1	2600	2
4	3	0	2700	1
4	3	1	3300	2
4	4	0	3400	2
5	0	1	3100	1
5	1	0	3300	1
5	1	1	4600	1
5	1	2	6300	2
5	2	0	4900	1
5	2	1	7000	1
5	2	2	9400	2
5	3	0	7900	1
5	3	1	11000	1
5	3	2	14000	1
5	4	0	13000	1
5	4	1	17000	1
5	4	2	22000	1
5	4	3	28000	2
5	4	4	35000	2
5	5	0	24000	1
5	5	1	35000	1
5	5	2	54000	1
5	5	3	92000	1
5	5	4	160000	1
5	5	5	>180000	

Table 3: Most probable number of organisms: table for multiple tube methods using 5 × 0.01g, 5 × 0.001g, 5 × 0.0001g**.**

0.01g	0.001g	0.0001g	MPN/100g	Category
0	0	0	<2000	
0	1	0	2000	2
1	0	0	2000	1
1	0	1	4000	2
1	1	0	4000	1
2	0	0	5000	1
2	0	1	7000	2
2	1	0	7000	1
2	1	1	9000	2
2	2	0	9000	1
3	0	0	8000	1
3	0	1	11000	1
3	1	0	11000	1
3	1	1	14000	2
3	2	0	14000	1
3	2	1	17000	2
3	3	0	17000	2
4	0	0	13000	1
4	0	1	17000	1
4	1	0	17000	1
4	1	1	21000	1
4	2	0	22000	1
5	0	0	23000	1
4	2	1	26000	2
4	3	0	27000	1
4	3	1	33000	2
4	4	0	34000	2
5	0	1	31000	1
5	1	0	33000	1
5	1	1	46000	1
5	1	2	63000	2
5	2	0	49000	1
5	2	1	70000	1
5	2	2	94000	2
5	3	0	79000	1
5	3	1	110000	1
5	3	2	140000	1
5	4	0	130000	1
5	4	1	170000	1
5	4	2	220000	1
5	4	3	280000	2
5	4	4	350000	2
5	5	0	240000	1
5	5	1	350000	1
5	5	2	540000	1
5	5	3	920000	1
5	5	4	1600000	1
5	5	5	>1800000	

Cefas Standard Operating Procedure – Enumeration of *Escherichia coli* in molluscan shellfish http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_E_coli_04_04_08.pdf Adapted from: ISO 7218:2007.

To calculate the most probable number (MPN), record the number of TBGA plate positives for each dilution. This gives a three figure tube combination number, which is used to calculate the MPN. MPN tube combinations fall into one of four categories. 95% of observed tube combinations fall in to category 1 with 4%, 0.9% and 0.1% in categories 2, 3 and 0 respectively. Both the category and MPN result can be determined from the MPN table (see Appendix 2) as follows:

- **For dilutions of neat, 10–1 and 10–2 use MPN Table 1.**
- **For dilutions of 10–1, 10–2 and 10–3 use MPN Table 2.**
- **For dilutions of 10–2, 10–3 and 10–4 use MPN Table 3.**
- **For greater dilutions use MPN Table 3 and multiply the result by the extra number of dilution factors.**

Where more than three dilutions have been tested for a sample, select the tube combination as stated in the following rules:

1. Select the combination of three consecutive dilutions having a category 1 profile to obtain the MPN index. If more than one combination having a category 1 profile is obtained, use the one with the highest number of positive tubes.

2. If no combination having a category 1 profile is available, use the one having a category 2 profile. If more than one combination having a category 2 profile is obtained, use the one with the highest number of positive tubes.

Results should be reported as the most probable number per 100g of shellfish. Negative samples should be reported as MPN <20/100g. Where the MPN tube combination is not given in the relevant table, the result should be reported as 'Void'.

Note: The 5-tube 3-dilution MPN table given in ISO 7218:2007 includes all category 1 and category 2 combinations, and some (but not all) category 3 combinations. A note is included in the standard that: "Before starting testing, it should be decided which category will be acceptable, that is, only 1, 1 and 2 or even 1, 2 and 3. When the decision to be taken on the basis of the result is of great importance, only category 1, or at most 1 and 2, results should be accepted. Category 0 results should be considered with great suspicion". Given that the NRL generic SOP will be referred to by official control laboratories, all of the category 3 combinations have been omitted from the version of the tables presented here.

Material Safety Data Sheets (MSDS)

1. Description: naturally or artificially contaminated live bivalve shellfish
2. IATA Characterisation: – *In the context of proficiency testing live bivalve shellfish are designated BIOLOGICAL SUBSTANCES, Division 6.2, Infectious substances Category B, UN3373.*

***Name*:**

Oysters (*Crassostrea gigas* and *Ostrea edulis*)	☒
Mussels (*Mytilus* spp.)	☐
Hard shell clams (*Mercenaria mercenaria*)	☐
Manila clams (*Tapes philippinarum*)	☐
King scallops (*Pecten maximus*)	☐
Queen scallops (*Aequipecten opercularis*)	☐
Cockles (*Cerastoderma edule*)	☐
Razor clams (*Ensis* spp.)	☐
Palourdes (*Tapes decussatus*)	☐

***Synonyms*:** Bivalve molluscan shellfish, bivalve molluscs, common or Latin names (see above)

3. Hazard
Live bivalve shellfish used in proficiency testing maybe naturally contaminated with a range of potentially pathogenic microorganisms and indicator organisms at the harvesting area or artificially bio-accumulated or spiked in the laboratory. If contamination occurs naturally in UK harvesting areas it is highly unlikely that contaminants will be other than ACDP Hazard Group 2 organisms present in human sewage or animal faeces. These may include *E. coli,* FRNA bacteriophage*,* norovirus, *Salmonella* spp., *Vibrio* spp. and rarely hepatitis A. Hazard Group 2 organisms may cause human disease and may be hazardous to persons working in the laboratory. Good microbiological practice should be observed to reduce risk to laboratory staff.

Artificially contaminated bivalve shellfish will contain microorganisms in addition to those potentially present naturally. The National Reference Laboratory uses standardized approaches to bio-accumulation of bivalve molluscs under controlled conditions for the following Hazard Group 2 organisms:

- **Non-pathogenic *Escherichia coli*** - strains of *E. coli* used in bio-accumulation are non-enterotoxigenic, non-enteropathogenic, non-enteroinvasive, non-enterohaemorrhagic and non-enteroaggregative.
- ***Salmonella* spp.** - excluding *Salmonella* Typhi and Paratyphi.
- **Norovirus -** genetically characterised genotypes of genogroup I and II human norovirus from faecal material.
- **Hepatitis A virus** - strain pHM175 43c (HM-175) vaccine strain HM 175 strain contains mutations involved in culture adaptation which enable it to grow well in culture and which attenuate its human pathogenicity.

The above are considered low risk for laboratory personnel, aerosol exposure has not been demonstrated.

Storage and handling
It is advised that samples of live bivalve shellfish are processed immediately, under certain circumstances however they maybe stored at 3±2°C for short periods. Samples must be processed in a laboratory environment that is suitable for handling microorganisms categorized as ACDP Hazard Group 2 or equivalent. Staff handling live bivalve shellfish should have undergone appropriate training to include handling infectious biological substances and opening and homogenization of shellfish.
Precautions to prevent accidental injury when opening (shucking) shellfish should be taken. Accidental injury through stabbing with shucking knifes or puncture wounds from shells are common. The use of personnel protective equipment including protective specialist gloves (Kevlar or chain mail), or semi-automated shucking machine (e.g. Florida Cracker), and wooden or plastic blocks to hold animals is recommended. Good personal hygiene and thorough washing of hands is required.

Disposal: Decontaminate before disposal by autoclaving or equivalent.

Note. The information and recommendations contained in this Materials Safety Data Sheet are complied from sources understood to be reliable, however we accept no responsibility for the accuracy, or reliability or for any loss of injury resulting from the use of this information. Equally emerging hazards may not be covered in this document.

European Community Reference laboratory
for monitoring bacteriological and viral
contamination of bivalve molluscs

Report on the Norovirus/Hepatitis A Ring Trial

CRL ring trial reference: RT 27 (NoV/HAV)

Cefas Weymouth Laboratory,
Barrack Road, The Nothe,
Weymouth, Dorset, DT4 8UB, UK
Telephone: +44 (0) 1305 206600
Fax: +44 (0) 1305 206601
E-mail: fsq@cefas.co.uk
Web site: **www.crlcefas.org**

Contents

1. Introduction 3
2. Proficiency testing samples 3
2.1 Sample preparation and distribution 3
2.2 Quality control at dispatch 3
3. Results 3
3.1 Confidentiality of results 3
3.2 Reference results 3
3.3 Analysis of results 4
4. Conclusion and discussion 5
4.1 General comments 5
4.2 Discussion 5
5. References 5

Tables

Table 1: Source of viruses and designation 3
Table 2: Taqman expected results of RT 27 ring trial material 3
Table 3: Participants' results for all lenticules (L1 – L6) 4

Appendix

Appendix I: Reference results 6
Appendix II: Participants' presence/absence results for each lenticules 7
Appendix III: Participants' GI and GII C_t values for each lenticule 8
Appendix IV: Participants' HAV C_t values for each lenticule 9
Appendix V: Participants' and reference quantities per lenticules for each target 10

1. Introduction

In December 2008 the CRL organised a distribution of laboratory constructed samples in lenticule format for the detection of norovirus and hepatitis A virus.

2. Proficiency testing samples

2.1 Sample preparation and distribution

Laboratory constructed lenticules (RT 27 L1 to RT 27 L6) were prepared following the method of Codd *et al* (1998) with minor modifications. Hepatitis A virus (HAV), NoV genogroup I and II (Table 1) were prepared in phosphate buffered saline (PBS) and added at a 1:5 ratio to lenticulating fluid. The inoculated lenticulating fluid was aliquoted onto parafilm in 25µl volumes and placed in a desiccating chamber at 3±2C for 1 week. After 1 week lenticules were transferred to < -15˚C. Samples were packaged according to IATA regulations and distributed to twenty-eight participating laboratories by Worldnet shipping on the 1st December 2008. On receipt, participants were requested to store the samples at <-15˚C prior to analysis during the week commencing the 8th December 2008.

Table 1: Source of viruses and designation

Sample description	Source	Sequence type
Norovirus genogroup I	Faecal material	GI.4 capsid type; 96.2% sequence homology to Chiba virus (AB022679)
Norovirus genogroup II	Faecal material	GII.4 capsid type; 99.7% sequence homology to Isumi strain (AB295790)
Hepatitis A	Laboratory reference strain	strain HM175/43c

2.2 Quality control at dispatch

Lenticules were tested prior to distribution to confirm virus presence and levels. Analyses were undertaken using CRL routine one-step RT-PCR method. The expected results and estimated quantities (geometric mean) for each lenticule are given in Table 2.

Table 2: Taqman™ expected results of RT 27 ring trial material

Sample	Norovirus GI	Norovirus GII	HAV
RT 27 – L1	-	+ (3.3×10^3)	-
RT 27 – L2	-	-	-
RT 27 – L3	+ (3.6×10^3)	-	+ (1.3×10^5)
RT 27 – L4	+ (2.6×10^3)	+ (1.7×10^3)	+ (1.2×10^6)
RT 27 – L5	-	-	+ (7.8×10^4)
RT 27 – L6	+ (4.9×10^3)	-	-

[a] Geometric mean quantities expressed as genome copies per lenticule in parentheses

3.0 Results

3.1 Confidentiality of results

Each laboratory was provided with a personal identification number to preserve anonymity.

3.2 Reference results

Reference analyses were performed by the CRL on lenticules stored at <-15˚C. Three randomly selected lenticules (L1-L6) were extracted in duplicate and RT-PCR (TaqMan™) was carried

out in triplicate. Box and whisker plots describing estimated target quantities derived from reference testing and expressed as genome copies per lenticule are given in Appendix I.

3.3 Analysis of results

Twenty-seven laboratories returned results. Participants' results (Table 3) were assessed as percentage relative sensitivity, specificity and accuracy for each determinant according to the following calculations:

Percentage relative sensitivity: $$\text{Relative sensitivity (SE)} = \frac{TP}{(TP+FN)} \times 100\%$$

Percentage relative specificity: $$\text{Relative specificity (SP)} = \frac{TN}{(TN+FP)} \times 100\%$$

Percentage relative accuracy: $$\text{Relative accuracy (AC)} = \frac{TP+TN}{N} \times 100\%$$

Where TP = true positives
FN = false negatives
FP = false positives
TN = true negatives
N = total number of tests

Note: Participants' results were expressed as percentage concordance with intended results generated by the CRL. In this assessment presence/absence data was used and no consideration of quantitative measurements (Ct values) was made.

Table 3. Participants' results for all lenticules (L1 - L6)

Lab ID number	GI			GII			HAV		
	SE	SP	AC	SE	SP	AC	SE	SP	AC
2	100	100	100	100	75	83	100	100	100
3	100	100	100	100	50	67	100	67	83
7	100	100	100	100	100	100	100	100	100
9	33	67	50	100	50	67	33	67	50
10	100	100	100	100	100	100	100	100	100
11	100	100	100	100	100	100	100	100	100
15	100	100	100	100	100	100	100	100	100
17	100	100	100	100	100	100	100	100	100
19	100	100	100	100	100	100	100	100	100
21	100	100	100	100	100	100	100	100	100
24	100	100	100	100	100	100	100	100	100
25	100	100	100	100	100	100	100	100	100
27	33	33	33	100	100	100	100	100	100
32	100	100	100	100	100	100	100	100	100
33	100	100	100	100	100	100	100	100	100
35	100	100	100	100	100	100	100	100	100
37	100	100	100	100	100	100	100	100	100
39	67	100	83	50	100	83	100	100	100
41	100	100	100	100	100	100	100	100	100
44	33	100	67	0	75	50	0	100	50
47	67	100	83	100	100	100	100	100	100

Lab ID number	GI			GII			HAV		
	SE	SP	AC	SE	SP	AC	SE	SP	AC
48	100	100	100	100	100	100	67	100	83
83	100	100	100	100	100	100	100	100	100
94	100	100	100	100	100	100	100	100	100
114	100	100	100	100	100	100	100	100	100
133	100	100	100	100	100	100	NE	NE	NE
146	67	100	83	100	100	100	100	100	100

NE - Not examined
Lab 44 –Reported NoV presence or absence, and did not differentiate between genogroups.
Lab 133 - Did not test for HAV.

4. Conclusion and discussion

4.1 General comments

Twenty-seven laboratories (17 NRLs and 10 non-NRL and third country laboratories) returned results. Laboratory 133 did not examine for HAV. Laboratory 44 used a non-discriminatory method for detection of NoV. Laboratory 113 did not return results. Results reported to the CRL are shown in Appendices I, II and III.

4.2 Discussion

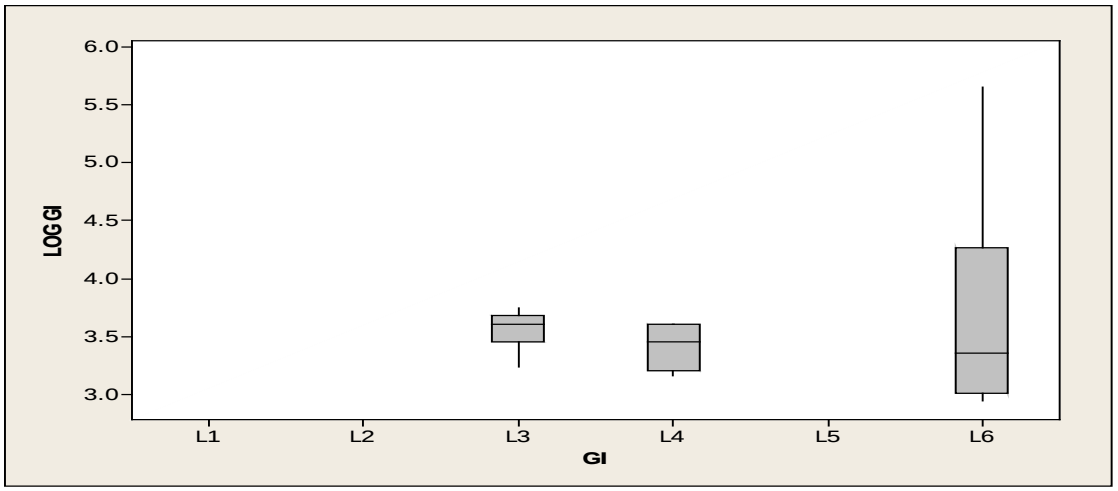
Approximately a third (67%) of participating laboratories obtained intended results for all lenticules, as determined by CRL reference designations. For individual viruses, 41%, 48% and 72% of laboratories returned intended results for norovirus GI, GII and hepatitis A respectively. One laboratory did not examine for HAV. The false positive reporting rates for GI, GII and HAV were 4%, 6% and 6% respectively. The false negative reporting rates for GI, GII and HAV were 11%, 6% and 11% respectively. Eighteen laboratories (67%) returned semi-quantitative data expressed at C_t values (Appendices II, III and IV). Seven laboratories returned quantitative data expressed as detectable genome copies per lenticule (Appendix V) .

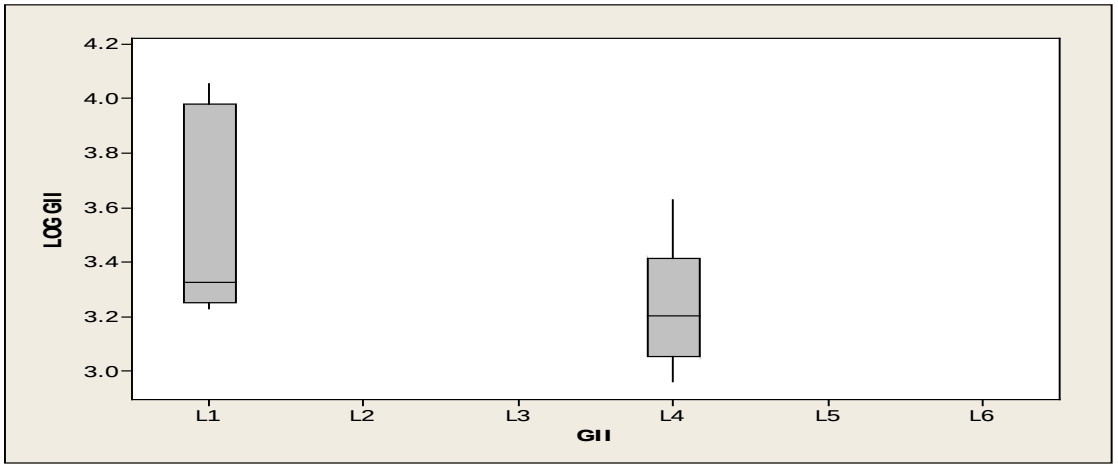
5 References

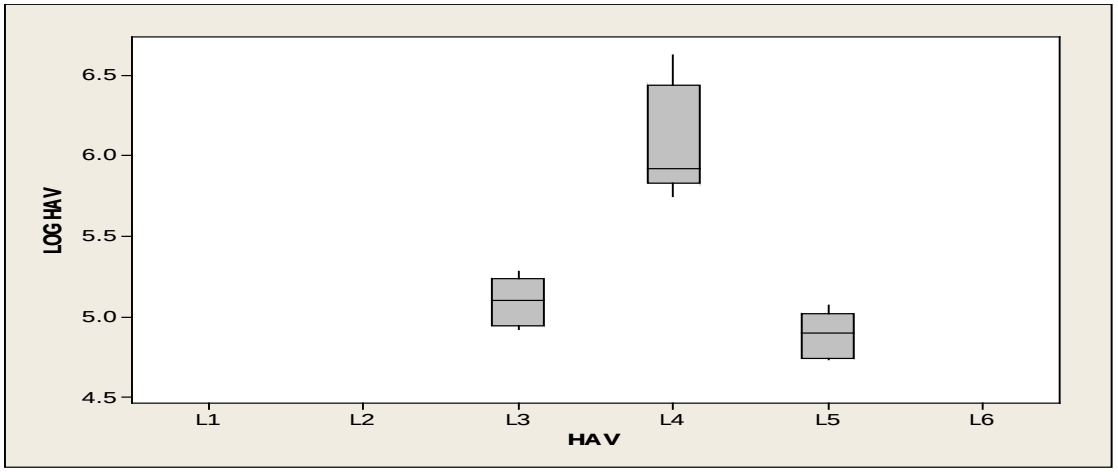
Codd AA, Richardson IR, Andrews N. 1998. Lenticules for the control of quantitative methods in food microbiology. J Appl Microbiol. **85(5)**:913–7.

Appendix I - Reference results

CRL Reference results displayed as box and whisker plots of detectable genome copies per 25µl lenticule.







Appendix II - Participants' presence/absence results for each lenticule

Lab ID	Lenticule 1			Lenticule 2			Lenticule 3			Lenticule 4			Lenticule 5			Lenticule 6		
	GI	GII	HAV	GI	GII	HAV	GI	GII	HAV	GI	GII	HAV	GI	GII	HAV	GI	GII	HAV
	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
2	-	+	-	-	-	-	+	-	+	+	+	+	-	+	+	+	-	-
3	-	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-
7	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
9	-	+	-	-	+	+	-	-	-	-	+	-	+	+	+	+	-	-
10	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
11	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
15	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
17	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
19	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
21	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
24	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
25	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
27	-	+	-	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-
32	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
33	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
35	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
37	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
39	-	-	-	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-
41	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
44	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
47	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-
48	-	+	-	-	-	-	+	-	+	+	+	+	-	-	-	+	-	-
83	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
94	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
114	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-
133	-	+	nt	-	-	nt	+	-	nt	+	+	nt	-	-	nt	+	-	nt
146	-	+	-	-	-	-	-	-	+	+	+	+	-	-	+	+	-	-

Yellow denotes false positives, Red denotes false negatives.

Cefas

Appendix III - Participants' reported GI and GII C_t values for each lenticule

Lab. ID.	Lenticule 1		Lenticule 2		Lenticule 3		Lenticule 4		Lenticule 5		Lenticule 6	
	NV GI	NV GII	NV GI	NV GII	NV GI	NV GII	NV GI	NV GII	NV GI	NV GII	NV GI	NV GII
2	-	+	-	-	+	-	+	+	-	+	+	-
3	-	34.26, 33.29	-	-	39.37, 39.37	37.55	40.67, 40.78	35.43, 34.91	-	-	40.7, 40.37	38.73, no Ct
7	-	+	-	-	+	-	+	+	-	-	+	-
9	-	+	-	+	-	-	-	+	+	+	+	-
10	-	37.59, 37.6	-	-	36.43, 36.29	-	36.82, 36.49	36.49, 35.03	-	-	37.09, 37.96	-
11	-	38.15	-	-	39.18	-	39.07	40.48	-	-	38.01	-
15	-	33	-	-	40	-	39	34	-	-	38	-
17	-	31.24	-	-	37.62	-	36.96	30.19	-	-	37.86	-
19	-	35.6	-	-	37	-	37.4	36.2	-	-	39.8	-
21	-	36.8	-	-	38.7	-	37.7	37.1	-	-	40	-
24	-	32.78	-	-	37.17	-	37.31	33.86	-	-	36.57	-
25	-	33.79, 33.84	-	-	39.69, 39.94	-	40.39, 40.11	37.49, 37.59	-	-	40.63, 40.28	-
27	-	45.8, 43.5	42.2, 41.9	-	45.4, 44.5	-	-	47.9, 43.4	50, 50.3	-	-	-
32	-	36.9	-	-	39.6	-	39.4	38	-	-	40.2	-
33	-	+	-	-	+	-	+	+	-	-	+	-
35	-	+	-	-	+	-	+	+	-	-	+	-
37	-	+	-	-	+	-	+	+	-	-	+	-
39	-	-	-	-	+	-	+	33, 35	-	-	-	-
41	-	31.32	-	-	36.62	-	37.37	31.78	-	-	36.21	-
44	-	-	-	-	+	+	-	-	-	-	-	-
47	-	+	-	-	+	-	+	+	-	-	-	-
48	-	35.1	-	-	39.9	-	40.5	34.9	-	-	41.9	-
83	-	34.8	-	-	35.8	-	36.5	35.3	-	-	35.3	-
94	-	29.47	-	-	34.66	-	33.77	28.99	-	-	33.4	-
114	-	30.34	-	-	33.09	-	33.39	31.4	-	-	33.44	-
133	-	36.82	-	-	36.55	-	34.34	36.7	-	-	36.9	-
146	-	36.63, 36.27	-	-	-	-	38.82, 39.71	36.35, 37.38	-	-	40.73, 40.72	-

Yellow denotes false positives, Red denotes false negatives.

Cefas

Appendix IV - Participants' reported HAV C_t values for each lenticule

Lab. ID.	Lenticule 1 HAV	Lenticule 2 HAV	Lenticule 3 HAV	Lenticule 4 HAV	Lenticule 5 HAV	Lenticule 6 HAV
2	-	-	+	+	+	-
3	-	39.62, no Ct	33.03, 32.31	32.3, 33.05	34.16, 33.73	-
7	-	-	+	+	+	-
9	-	+	-	-	+	-
10	-	-	31.67, 31.82	31.54, 31.79	32.58, 33.02	
11	-	-	36.14	36.29	37.09	-
15	-	-	36	34.5	35.5	-
17	-	-	31.01	31.85	32.77	-
19	-	-	32.3	31.8	33.4	-
21	-	-	35.7	35.4	37.8	-
24	-	-	+	+	+	-
25	-	-	31.42, 31.95	32.72, 32.48	33.39, 33.28	-
27	-	-	44.5, 43.4	46.3, 43.4	45.1, 45.6	-
32	-	-	31.1	30.9	32.4	-
33	-	-	+	+	+	-
35	-	-	+	+	+	-
37	-	-	+	+	+	-
39	-	-	27, 29	28, 30	31, 32	-
41	-	-	29.27	29.87	30.14	-
44	-	-	-	-	-	-
47	-	-	33.9	33.6	35.4	-
48	-	-	40.3	39.3	-	-
83	-	-	31.9	32.1	33.3	-
94	-	-	25.26	25.96	26.95	-
114	-	-	27.03	26.36	27.85	-
133	nt	nt	nt	nt	nt	nt
146	-	-	34.24, 34.54	33.48, 33.18	35.88, 36.08	-

Yellow denotes false positives, Red denotes false negatives.

Appendix V – Participants' and reference quantities per lenticule for each target.

Lenticule 1 - GII

Lenticule 3 - GI

Lenticule 3 - HAV

Lenticule 4 - GI

Lenticule 4 - GII

Lenticule 4 - HAV

Lenticule 5 – HAV

Lenticule 6 - GI

Community Reference Laboratory (CRL) Proficiency Testing Schemes

Report on the *Vibrio parahaemolyticus* ring trial 2009

CRL ring trial reference number: RT 29

Sample numbers: RT29.1; RT29.1; RT29.2; RT29.3; RT29.4;

Contents

1 Introduction 2
2 Proficiency testing samples 2
2.1 Sample preparation and distribution 2
2.2 Quality control at dispatch 2
3 Results 2
3.1 Confidentiality of results 2
3.2 Reference results 2
3.3 Analysis of results 3
4 Summary 3
5 References 4

Tables

Table 1. Source of *Vibrio* spp. strains 2
Table 2. *Vibrio* spp. reference range and PCR results 2
Table 3. Participants' results for RT29.1 Vial 1 5
Table 4. Participants' results for RT29.2 Vial 2 6
Table 5. Participants' results for RT29.3 Vial 3 7
Table 6. Participants' results for RT29.4 Vial 4 8

Appendices

Methods used for enumeration and detection of the pathogenic markers 9

Figures

Figure 1. *toxR* PCR 10
Figure 2. *tdh* PCR 10
Figure 3. *trh* PCR 10
Figure 4. Participants' mean quantitative cfu/g for vial 1 11
Figure 5. Participants' mean quantitative cfu/g for vial 2 11
Figure 6. Participants' mean quantitative cfu/g for vial 3 12
Figure 7. Participants' mean quantitative cfu/g for vial 4 12

1. Introduction

In March 2009 the CRL organised a distribution of freeze-dried cultures for the enumeration and determination of potential pathogenic *V. parahaemolyticus*.

2. Proficiency testing samples

2.1 Sample preparation and distribution

Four samples from the Cefas *Vibrio* spp. reference strain bank (Table 1) were streaked onto non-selective marine agar (MA). Plates were incubated for 18-24hrs at 30±2°C. Following visual purity checks 2-5 colony forming units (cfu) were inoculated into 2 x 100ml alkaline salt peptone water (ASPW) and incubated for 18-24hrs at 30±2°C. After incubation samples were centrifuged at 2000rpm for 20min. The supernatant was discarded and the pellets were resuspended in 15ml of mist desiccant containing ASPW. Each strain was designated a sample code, Vial 1 to 4. Aliquots of 0.2ml were added to sterile vials and freeze dried. Once freeze-drying was complete vials were stored at 3±2°C until distribution. Samples were packaged according to IATA regulations and distributed to twenty-two participating laboratories by Worldnet shipping on the 9th March 2009. On receipt, participants were requested to store the samples at 3±2°C prior to analysis during the week commencing the 16th March 2009.

Table 1: Source of *Vibrio* spp. strains

Sample ID	Reference bank designation	Source of strain	Presence of *tdh/trh*	Reference bank code
RT29. 1	*V. parahaemolyticus*	Clinical	*tdh* only	V05/65
RT29.2	*V. parahaemolyticus*	Clinical	*tdh* and *trh*	V05/14
RT29. 3	*V. alginolyticus*	Reference	No	NCTC 12160
RT29.4	*V. parahaemolyticus*	Environmental	*trh* only	V05/70

2.2 Quality control at dispatch

Samples were tested prior to distribution to confirm the reference strain bank designation using CRL standard procedures for detection of *V. parahaemolyticus*, with additional biochemical tests to confirm the identification of *V. alginolyticus*. In addition, polymerase chain reaction (PCR) was used to detect *toxR* and the haemolysin genes *tdh* and *trh* following the methods of Kim *et al* (1999) and Tada *et al* (1992) with minor modifications.

3.0 Results

3.1 Confidentiality of results

Each laboratory was provided with a personal identification number to preserve anonymity.

3.2 Reference results

Reference analyses were performed by the CRL on 4 randomly selected vials per batch to determine the species type and levels (cfu/g). The reference results are summarised in Table 2 and included in Figures 4 - 7. The presence of *tdh* and *trh* was determined using PCR as described above (Figure 1 - 3).

Table 2: *Vibrio* spp. ring trial expected reference range and PCR results

Sample ID	Reference range cfu/g	*toxR*	*tdh*	*trh*
RT29. 1	$2.6 \times 10^5 – 2.2 \times 10^6$	+	+	-
RT29. 2	$2.2 \times 10^3 – 3.2 \times 10^5$	+	+	+
RT29. 3	$8.0 \times 10^4 – 1.2 \times 10^6$	-	-	-
RT29. 4	$5.6 \times 10^5 – 1.9 \times 10^6$	+	-	+

3.3 Analysis of results

Twenty-two laboratories returned results to the CRL. Nine (41%) used methods that enabled detection of *tdh* and *trh* genes. Participants' results are shown in Tables 3 to 8 presumptive false positive, negative and uncertain results are identified in yellow, red and grey respectively. Results are provided for information only. No performance assessments were carried out. Laboratories reporting quantitative results are shown in Figures 4 - 7 with reference results included.

3.3.1 RT29.1 Vial 1 [CRL expected result *V. parahaemolyticus* present; *tdh* positive and *trh* negative]

Fourteen laboratories (64%) identified *V. parahaemolyticus* in both replicates. Two laboratories identified *V. parahaemolyticus* in a single replicate. Laboratories 22, 35, 76, 83, 85 and 113 reported the absence of *V. parahaemolyticus* with 1 identifying *V. vulnificus*. Nine laboratories carried out tests for *tdh* and/or *trh.* Three detected *tdh* positive and *trh* negative. Laboratory 44 reported the absence of *tdh* and laboratories 17, 19, 33, 68 and 137 reported the presence of *trh.* Nine laboratories reported quantitative results within the range of $2.0 \times 10^2 – 4.7 \times 10^5$.

3.3.2 RT29.2 Vial 2 [CRL expected result *V. parahaemolyticus* present; *tdh* and *trh* positive].

Seventeen laboratories (77%) identified *V. parahaemolyticus* in both replicates. One laboratory identified *V. parahaemolyticus* in a single replicate. Laboratories 76, 85 and 113 reported the absence of *V. parahaemolyticus.* Eleven laboratories applied tests for *tdh* and/or *trh* with 5 detecting *tdh* and *trh* positive. Laboratories 7, 32, 35 and 68 reported the absence of *trh* and laboratory 44 reported the absence of *tdh* and *trh.* Thirteen laboratories reported quantitative results within the range of $5.0 \times 10^0 – 4.2 \times 10^5$.

3.3.3 RT29.3 Vial 3 [CRL expected result *V. parahaemolyticus* not detected (sample *V. alginolyticus* NCTC12160 *)*; *tdh* and *trh* negative]

Eight laboratories (36%) identified the absence of *V. parahaemolyticus* in both replicates, 5 laboratories reported the presence of *V. alginolyticus*. Three laboratories identified the absence of *V. parahaemolyticus* in a single replicate of which 2 identified *V. alginolyticus.* Laboratories 22, 33, 68, 85, 124, 137 and 140 reported the presence of *V. parahaemolyticus.* Nine laboratories applied tests for *tdh* and/or *trh* with 5 reported absence of *tdh* and *trh* negative. Laboratories 35, 68 and 137 reported the presence of *trh.* Nine laboratories reported quantitative results within the range of $1.5 \times 10^2 – 1.2 \times 10^6$.

3.3.4 RT29.4 Vial 4 [CRL expected result *V. parahaemolyticus* present*, tdh* negative, *trh* positive]

Eighteen laboratories (82%) identified *V. parahaemolyticus* in both replicates. One laboratory identified *V. parahaemolyticus* in a single replicate. Laboratories 76 reported the absence of *V. parahaemolyticus.* Eleven laboratories applied tests for *tdh* and/or *trh* with 9 reporting *tdh* negative and *trh* positive. Laboratory 44 reported the absence of *trh.* Thirteen laboratories reported quantitative results within the range of $2.0 \times 10^2 – 4.6 \times 10^6$.

4 Summary

Of the 22 laboratories returned results for this ring trial. Forty one % of laboratories returned results corresponding to CRL expected results for presence / absence of *V. parahaemolyticus* in the 4 vials received. Eight laboratories detected the presence of *V. alginolyticus* in vial 3. A number of laboratories reported *V. parahaemolyticus* in vials where the presence of this organism was not anticipated. Further analysis of a limited number of reference vials stored at the CRL were unable to identify cross contamination of vial 3 using cultural and PCR methods. However the potential for laboratory cross contamination cannot be ruled out. Quantitative results were returned by between 13 and 14 laboratories dependent upon sample. Levels of *Vibrio* spp. were highly variable with over 4 $\log_{10}$ variation in participants resulted observed.

Eleven laboratories used various methods that enabled detection of *tdh* and *trh* (see Appendix I). The majority of laboratories applying these tests assigned the presence or absence of both *tdh* and *trh* in

accordance with the CRL tested results. One laboratory (Lab 44) reported duplicate *tdh* negative results for vials 1 and 2, and 3 laboratories (Lab 7, 32 and 44) reported *trh* as negative in vials 2 and 4. Overall the recognition of the *tdh* gene by laboratories applying the test was in accordance with CRL predicted and participants' results; overall accuracy rate ≈95%.

A number of laboratories (7, 12, 19, 32, 33, 35, 44, 68 and 137) reported *trh* results that differed from those anticipated by the CRL. These results fell into two categories, laboratories that did not detect the *trh* gene in vials 2 and 4, and those that detected *trh* in vials 1 and 3. For vials 2 and 4 most laboratories undertaking the tests assigned presence of *trh* genes in accordance with CRL predicted results; accuracy rate ≈76%. It has been reported that *trh* genes cluster into two main subgroups, *trh1* and *trh2*, which share 84% identity (Kishishita *et al* 1992). In addition Gonzalez-Escalona *et al* (2006) reported a *trh*-like gene of *V. alginolyticus* isolated from Alaskan oysters that shared 98% sequence homology with the *trh2* gene of *V. parahaemolyticus*. Methods of Tada *et al* (1995) used to characterise reference materials at the CRL prior to distribution detect *trh1* only. Subsequent tests on stored material appropriate for detection of *trh2* (Nordstrom *et al* 2006; Gonzalez-Escalona *et al* 2006) were negative however its presence cannot be ruled out and thus it was not possible to assign accuracy rates of *trh* detection.

Following analysis of results, and after discussion at the 8th workshop of NRL in Spain 2009 participants were requested to provide information on the methods used in RT29. Where information was provided it was noted that a number of method of standard methods (ISO, NMKL, BAM) and non-standard methods were in use for identification and enumeration of *V. parahaemolyticus*. Similarly a wide variety of published and laboratory developed procedures were used for detection of pathogenic principles. These are summarized in Appendix I.

The outcomes of this ring trial highlight the urgent need for standardization of fit-for-purpose methods for the detection and enumeration of potentially pathogenic *Vibrio* spp. for use in European bivalve shellfish.

5 References

Gonzalez-Escalona N., Blackstone G. M. And DePaola A. 2006) Charcteristaion of a *Vibrio alginolyticus* strain, isolated from Alaskan oysters carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (trh) of *Vibrio parahaemolyticus*. Applied and Environmental Microbiology 72, 12 7925-7929.

Kim, Y.B., J. Okuda, C. Matsumoto, N. Takahashi, S. Hashimoto, and M. Nishibuchi. 1999. Identification of *V. parahaemolyticus* strains at the species level by PCR targeted to the *ToxR* gene. *Journal of Clinical Microbiology*, 37:1173-1177.

Nordstrom J.L., Rangdale R.E. *,. Vickery M.C.L,. Phillips A.M.B,. Watt S and DePaola A. 2006 Identification and enumeration of potentially virulent *Vibrio parahaemolyticus* using an alkaline phosphatase labeled DNA probe targeting *trh" Journal of Food Protection,* 69:2770-2772.

Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M, and Takeda Y. 1992. Detection of the thermostable direct haemolysin gene (*tdh*) and the thermostable direct haemolysin-related haemolysin gene *trh*) of *V. parahaemolyticus* by polymerase chain reaction. Mol.Cell Probe 6: 477-487.

McCarthy S, DePaola A, Kaysner C, Hill W and Cook D. 2000. Direct plating procedure for the enumeration of total and pathogenic *Vibrio parahaemolyticus* in oyster meats. FDA/Gulf Coast Seafood Laboratory protocol Vp-ISSC-3. issue 4/03/00.

Table 3. Participants' results for RT29.1 Vial 1

[expected result *V. parahaemolyticus* present; *tdh* positive and *trh* negative]

Lab ID	Replicate 1 Sample result	tdh	trh	cfu/g	Replicate 2 Sample result	tdh	trh	cfu/g
7	*V. parahaemolyticus*	+	-	1.2×10^5	*V. parahaemolyticus*	+	-	2.5×10^5
10	*V. parahaemolyticus*	+	-	4.7×10^5	*V. parahaemolyticus*	+	-	4.7×10^5
17	*V. parahaemolyticus*	+	+	1.7×10^3	*V. parahaemolyticus*	+	+	NE
19	*V. parahaemolyticus*	+	+	>100	*V. parahaemolyticus*	+	+	>100
22	No *V. parahaemolyticus* present	NE	NE	NE	No *V. parahaemolyticus* present	NE	NE	NE
32	*V. parahaemolyticus*	+	-	2.9×10^5	*V. parahaemolyticus*	+	-	1.9×10^5
33	*V. parahaemolyticus*	+	+	NE	*V. parahaemolyticus*	+	+	NE
35	*V. vulnificus*	NE	NE	<10	*V. parahaemolyticus*	+	-	<10
44	*V. parahaemolyticus*	-	-	6.2×10^2	*V. parahaemolyticus*	-	NE	6.6×10^2
48	*V. parahaemolyticus*	NE	NE	4.6×10^2	*V. parahaemolyticus*	NE	NE	4.6×10^2
68	*V. parahaemolyticus*	+	+	1.0×10^5	*V. parahaemolyticus*	+	+	8.0×10^4
76	Other species	NE	NE	NE	Other species	NE	NE	NE
83	Negative	NE	NE	NE	Negative	NE	NE	NE
85	*V. parahaemolyticus* not detected	NE	NE	NE	*V. parahaemolyticus* not detected	NE	NE	NE
89	-	NE	NE	NE	-	NE	NE	NE
90	*V. parahaemolyticus*	NE	NE	7.0×10^4	*V. parahaemolyticus*	NE	NE	2.0×10^5
98	*V. parahaemolyticus*	NE	NE	3.9×10^5	*V. parahaemolyticus*	NE	NE	3.3×10^5
113	No *V. parahaemolyticus*	NE	NE	NE	NE	NE	NE	NE
115	*V. parahaemolyticus*	NE	NE	NE	NE	NE	NE	NE
124	*V. parahaemolyticus*	NE	NE	NE	NE	NE	NE	NE
137	*V. parahaemolyticus*	+	+	7.2×10^2	*V. parahaemolyticus*	+	+	5.6×10^2
140	*V. parahaemolyticus*	NE	NE	>11000	*V. parahaemolyticus*	NE	NE	>11000

NE – Not examined
Yellow denotes false positive, Red denotes false negative, Grey denotes uncertain result (see summary above)

Table 4. Participants' results for RT29.2 Vial 2

[expected result *V. parahaemolyticus* present; *tdh* and *trh* positive].

Lab ID	Replicate 1 Sample result	tdh	trh	cfu/g	Replicate 2 Sample result	tdh	trh	cfu/g
7	*V. parahaemolyticus*	+	-	3.7×10^4	*V. parahaemolyticus*	+	-	5.6×10^3
10	*V. parahaemolyticus*	+	+	3.7×10^3	*V. parahaemolyticus*	+	+	3.7×10^3
17	*V. parahaemolyticus*	+	+	7.3×10^2	*V. parahaemolyticus*	+	+	NE
19	*V. parahaemolyticus*	+	+	>100	*V. parahaemolyticus*	+	+	>100
22	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
32	*V. parahaemolyticus*	+	-	4.2×10^5	*V. parahaemolyticus*	+	+	3.6×10^5
33	*V. parahaemolyticus*	+	+	NE	*V. parahaemolyticus*	+	+	NE
35	*V. parahaemolyticus*	+	+	9.2×10^3	*V. parahaemolyticus*	+	-	4.3×10^3
44	*V. parahaemolyticus*	-	-	2.9×10^2	*V. parahaemolyticus*	-	-	3.1×10^2
48	*V. parahaemolyticus*	NE	NE	1.1×10^3	*V. parahaemolyticus*	NE	NE	2.4×10^3
68	*V. parahaemolyticus*	+	-	2.7×10^4	*V. parahaemolyticus*	+	-	2.4×10^4
76	Other species	NE	NE	NE	Other species	NE	NE	NE
83	Positive	+	NE	NE	Positive	+	NE	NE
85	*V. parahaemolyticus* not detected	NE	NE	NE	*V. parahaemolyticus* not detected	NE	NE	NE
89	Not detected	NE	NE	<1	Not detected	NE	NE	<1
90	*V. parahaemolyticus*	NE	NE	2.0×10^3	*V. parahaemolyticus*	NE	NE	8.0×10^2
98	*V. parahaemolyticus*	NE	NE	5.6×10^4	*V. parahaemolyticus*	NE	NE	1.1×10^5
113	*V. parahaemolyticus* not detected	NE	NE	NE	NE	NE	NE	NE
115	*V. parahaemolyticus*	NE	NE	NE	NE	NE	NE	NE
124	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
137	*V. parahaemolyticus*	+	+	<10	*V. parahaemolyticus*	+	+	<10
140	Presumptive *V. parahaemolyticus*	NE	NE	>11000	Presumptive *V. parahaemolyticus*	NE	NE	2.4×10^3

NE – Not examined
Red denotes false negative, Grey denotes uncertain result (see summary above)

Table 5. Participants' results for RT29.3 Vial 3

[expected result *V. parahaemolyticus* not detected (sample *V. alginolyticus)*; *tdh* and *trh* negative]

Lab ID	Replicate 1 Sample result	tdh	trh	cfu/g	Replicate 2 Sample result	tdh	trh	cfu/g
7	Non *V. parahaemolyticus*	-	-	3.0 x 10^3	Non *V. parahaemolyticus*	-	-	1.3 x 10^4
10	*V. alginolyticus*	-	-	3.8 x 10^5	*V. alginolyticus*	-	-	3.8 x 10^5
17	*V. parahaemolyticus*	-	+	<10	*V. parahaemolyticus*	-	+	<10
19	No *V. parahaemolyticus, Past. Multocida*	-	-	>100	No *V. parahaemolyticus, Past. Multocida*	-	-	>100
22	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
32	*V. alginolyticus*	-	-	1.0 x 10^6	*V. alginolyticus*	-	-	1.2 x 10^6
33	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
35	*V. alginolyticus*	NE	NE	2.9 x 10^3	*V. parahaemolyticus*	-	+	<1000
44	*V. alginolyticus*	-	-	1.1 x 10^4	*V. alginolyticus*	-	-	1.0 x 10^4
48	*V. alginolyticus*	NE	NE	<300	*V. alginolyticus*	NE	NE	<300
68	*V. parahaemolyticus* + *Vibrio* spp.	-	+	1.2 x 10^5	*V. parahaemolyticus* + *Vibrio* spp.	-	+	1.1 x 10^5
76	Other species	NE	NE	NE	Other species	NE	NE	NE
83	Positive	-	NE	NE	Positive	-	NE	NE
85	*V. parahaemolyticus*	NE	NE	<20	*V. parahaemolyticus*	NE	NE	<20
89	-	NE	NE	NE	-	NE	NE	NE
90	*V. alginolyticus*	NE	NE	>1000	*V. alginolyticus*	NE	NE	>1000
98	*V. alginolyticus* and *V. parahaemolyticus*	NE	NE	2.8 x 10^5	*V. alginolyticus* and *V. parahaemolyticus*	NE	NE	5.4 x 10^5
113	*V. parahaemolyticus* not detected	NE	NE	NE	NE	NE	NE	NE
115	*V. alginolyticus*	NE	NE	NE	NE	NE	NE	NE
124	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
137	*V. parahaemolyticus*	-	+	<10	*V. parahaemolyticus*	-	+	<10
140	*V. parahaemolyticus*	NE	NE	<3	*V. parahaemolyticus*	NE	NE	<3

NE – Not examined
Yellow denotes false positive, ,Grey denotes uncertain result (see summary above)

Table 6. Participants' results for RT29.4 Vial 4

[expected result *V. parahaemolyticus* present, *tdh* negative, *trh* positive]

Lab ID	Replicate 1 Sample result	tdh	trh	cfu/g	Replicate 2 Sample result	tdh	trh	cfu/g
7	*V. parahaemolyticus*	-	+	1.2×10^5	*V. parahaemolyticus*	-	+	1.6×10^6
10	*V. parahaemolyticus*	-	+	4.9×10^6	*V. parahaemolyticus*	-	+	4.9×10^6
17	*V. parahaemolyticus*	-	+	4.2×10^3	*V. parahaemolyticus*	-	+	4.2×10^3
19	*V. parahaemolyticus*	-	+	>100	*V. parahaemolyticus*	-	+	>100
22	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
32	*V. parahaemolyticus*	-	+	1.7×10^6	*V. parahaemolyticus*	-	+	1.5×10^6
33	*V. parahaemolyticus*	-	+	NE	*V. parahaemolyticus*	-	+	NE
35	*V. parahaemolyticus*	-	+	2.2×10^4	*V. parahaemolyticus*	-	+	2.0×10^4
44	*V. parahaemolyticus*	-	-	2.9×10^4	*V. parahaemolyticus*	-	-	3.0×10^4
48	*V. parahaemolyticus*	NE	NE	1.1×10^4	*V. parahaemolyticus*	NE	NE	1.1×10^4
68	*V. parahaemolyticus*	-	+	9.0×10^5	*V. parahaemolyticus*	-	+	8.5×10^5
76	Other species	NE	NE	NE	Other species	NE	NE	NE
83	Positive	-	NE	NE	Positive	-	NE	NE
85	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
89		NE	NE	<1		NE	NE	<1
90	*V. parahaemolyticus*	NE	NE	1.0×10^4	*V. parahaemolyticus*	NE	NE	1.0×10^4
98	*V. parahaemolyticus*	NE	NE	5.5×10^5	*V. parahaemolyticus*	NE	NE	4.6×10^5
113	*V. parahaemolyticus*	NE	NE	NE	NE	NE	NE	NE
115	NE	NE	NE	NE	NE	NE	NE	NE
124	*V. parahaemolyticus*	NE	NE	NE	*V. parahaemolyticus*	NE	NE	NE
137	*V. parahaemolyticus*	-	+	9.0×10^3	*V. parahaemolyticus*	-	+	5.9×10^3
140	*V. parahaemolyticus*	NE	NE	>11000	*V. parahaemolyticus*	NE	NE	>11000

NE – Not examined
Red denotes false negative

Appendix I. Methods used for enumeration and detection of the pathogenic markers of *V. parahaemolyticus*.

Lab ID	Enumeration of *vibrio* species	Detection of pathogenic markers
7	ISO 8914 and direct plating[4]	Cefas PCR SOP[1]
10	ISO/TS 21872 - Part 1	Cefas PCR SOP and hybridisation[7]
17	-	-
19	Cefas SOP 1333	-
22	ISO/TS 21872 - Part 1	-
32	Cefas SOP 1333[3]	-
33	Cefas SOP 1333[3]	PCR target *toxR, tdh* and *trh* genes[2]
35	Direct plating[4]	Cefas PCR SOP[1]
44	NMKL method no.156	-
48	ISO/TS 21872	-
68	FDA Bacteriological Analytical Manual	PCR target *toxR, tdh* and *trh* genes[6]
76	SP-VG-M006	-
83	NMKL method no.156	-
85	-	-
89	FDA Bacteriological Analytical Manual	-
90	ISO/TS 21872 and direct plating[4]	-
98	ISO/TS 21872 - Part 1and 2	-
113	ISO/TS 21872 - Part 2	Real-time PCR[5]
115	ISO/TS 21872 - Part 1	-
124	ISO/TS 21872 - Part 1	-
137	ISO/TS 21872 - Part 1	Real-time PCR target *toxR, tdh, trh1* and *trh2* genes[2]
140	FDA Bacteriological Analytical Manual	-

[1] Derived from Kim *et al* (1999) *toxR* and *tdh* , Tada *et al* (1999) for *trh*
[2] No further information supplied
[3] Derived from ISO TS 21872-1
[4] Direct plating onto TCBS followed by biochemical confirmation of sucrose negative colonies
[5] Derived from Bej *et al* (1999) for *tdh*
[6] Derived from Bej *et al* (1999) for *tlh* and Kim *et al* 1999) for *toxR*. *Trh* and *tdh* positives derived from Tada *et al* (1992) sequences R2-R6 for *trh* gene and the sequences D1-D2 for *tdh* gene.
[7] Derived from Kim *et al* (1999) *toxR* and *tdh* **,** Tada *et al* (1999) for *trh* , Nordstrom *et al* (2006) and McCarthy *et al* (2000).

Figure 1. Tox R PCR

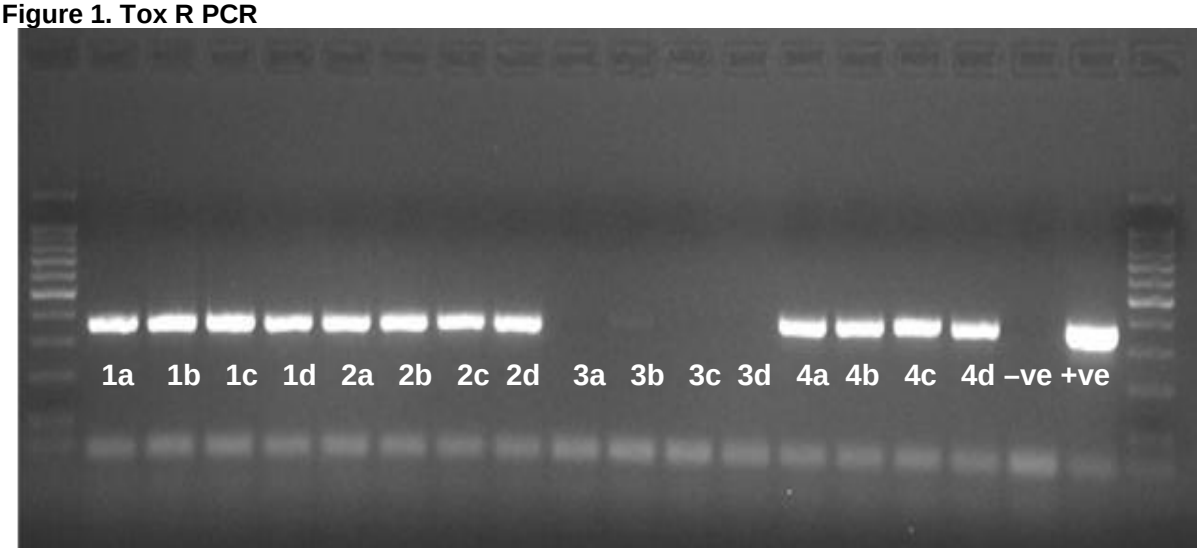


Figure 2. *tdh* PCR

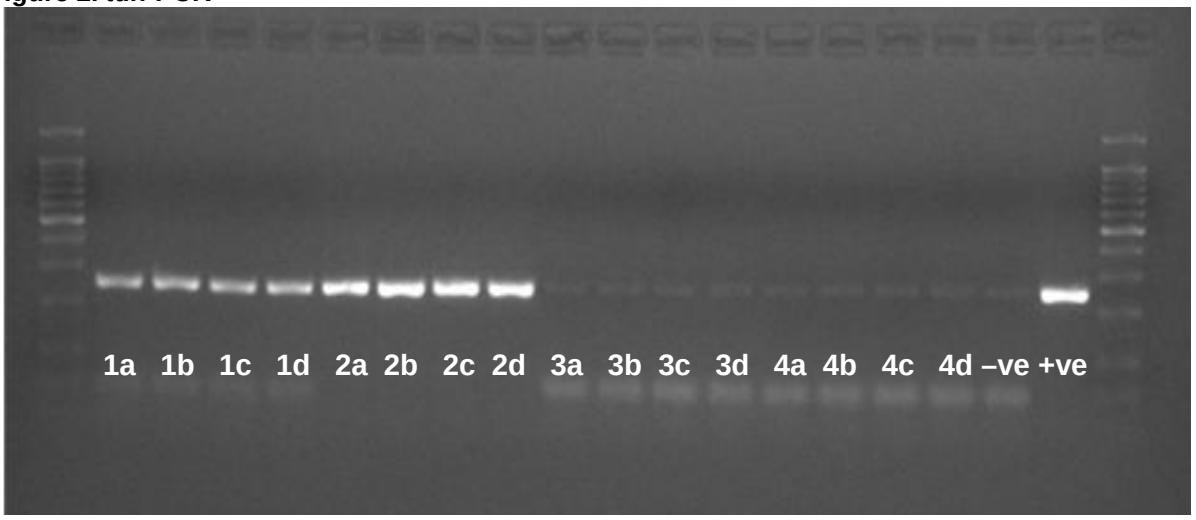


Figure 3. *trh* PCR

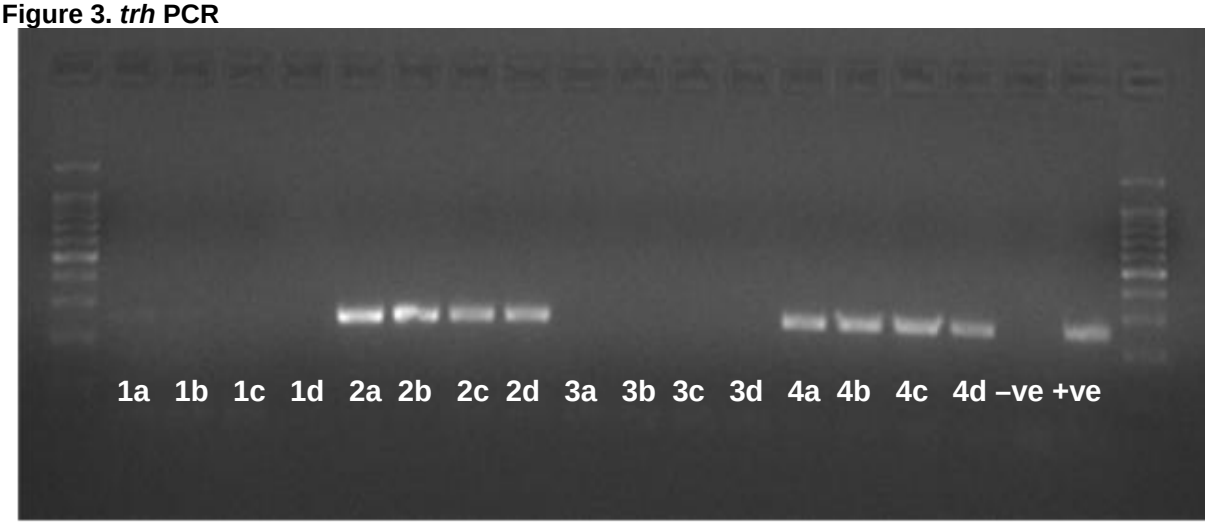


Figure 4. Participants' mean quantitative (cfu/g) for vial 1.

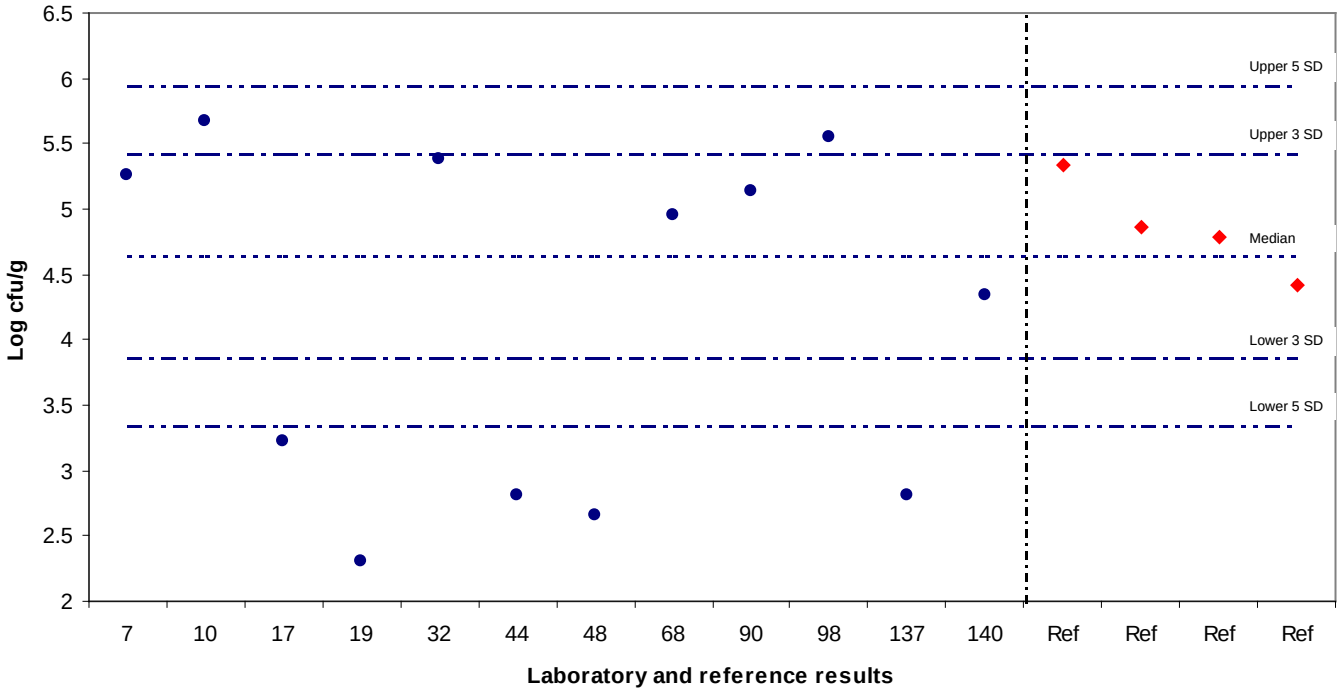


Figure 5. Participants' mean quantitative (cfu/g) for vial 2.

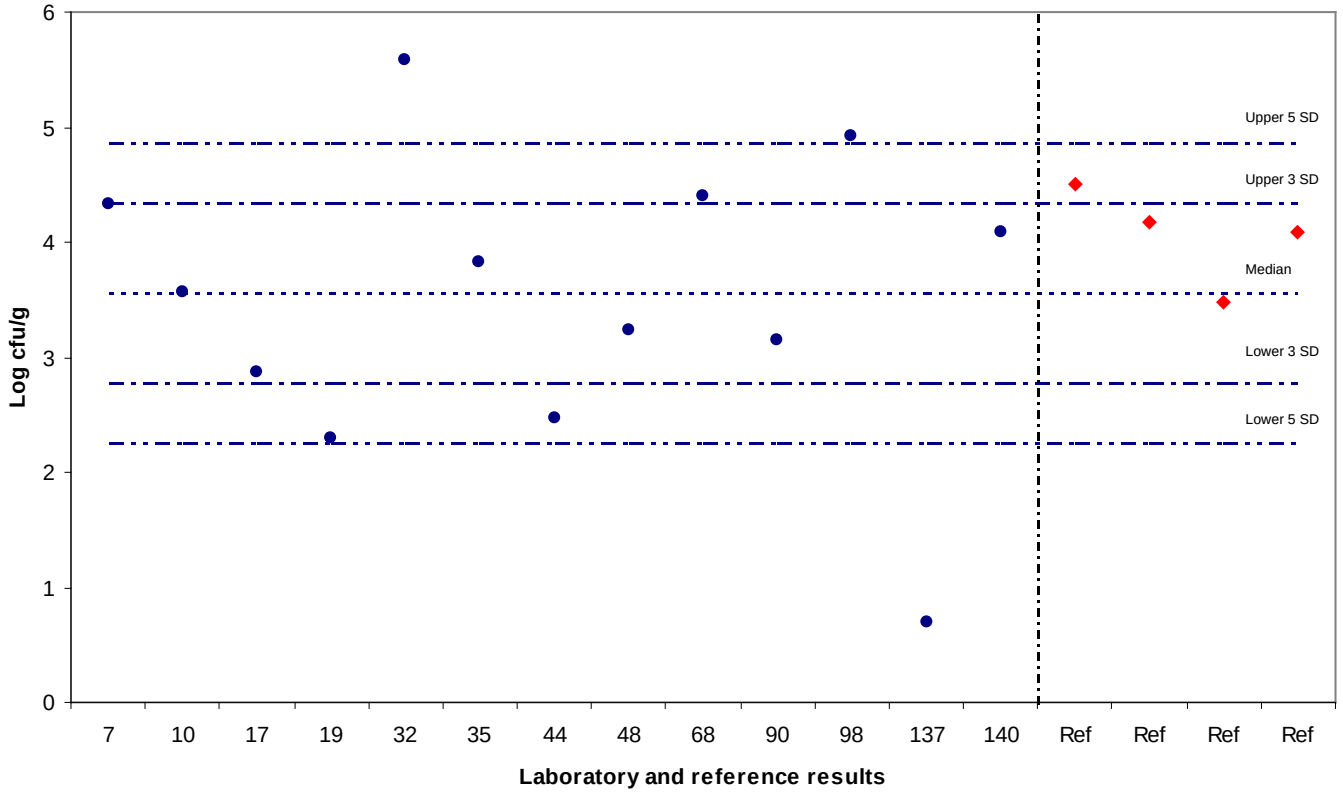


Figure 6. Participants' mean quantitative (cfu/g) for vial 3.

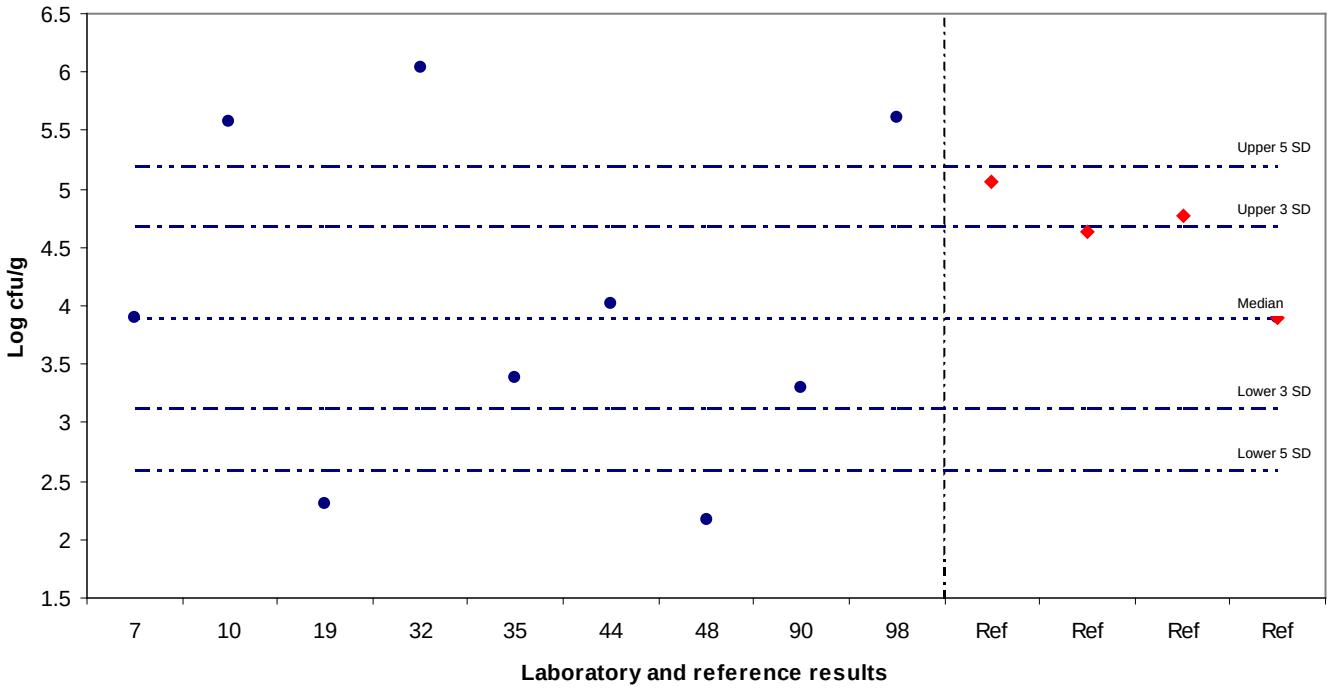
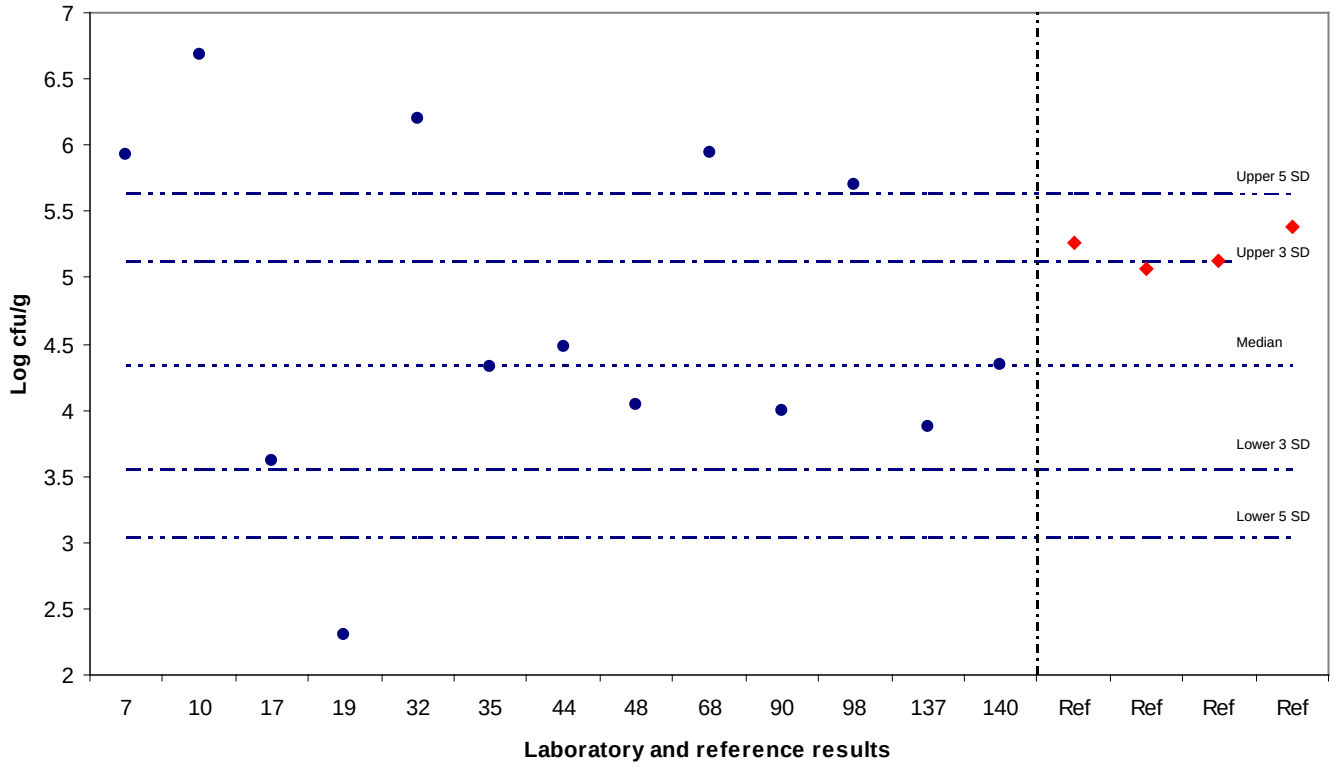


Figure 7. Participants' mean quantitative (cfu/g) for vial 4.



Dr Rachel Rangdale … Date……25.03.10
CRL Co-ordinator

Dr David Lees… Date……25.03.10
CRL Director